# DELAYED-TYPE HYPERSENSITIVITY AND ACQUIRED RESISTANCE IN LISTERIOSIS

Thesis submitted for the degree of Doctor of Philosophy

by

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# STATEMENT

The histological sections were prepared by Mr. R.G. Hill. The remainder of the work described in this thesis was carried out by the candidate.

Val Anderson

My remarks are..., I trust, true on the whole; though I do not pretend to say that they are void of mistake, or that a more nice observer might not make many additions, since subjects of this kind are inexhaustible.

Gilbert White.

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# INTRODUCTION

## I. AIM OF THE INVESTIGATION

When a mammalian host survives an encounter with a microbial parasite, this infection generally induces certain specific responses in the host:

(a) the serum and body fluids may be found to contain antibodies against one or more constituents of the parasite;

(b) delayed-type hypersensitivity may be demonstrable on challenge with the parasite or its products;

(c) the mononuclear phagocytes of the convalescent host may possess an enhanced ability to destroy the parasite;

(d) acquired resistance to subsequent attacks of the parasite may be present.

To dissect the nature of this acquired resistance and decide the roles played in it by other manifestations of the immune response is a major concern of immunology.

The knowledge that one attack of an infectious disease often confers immunity against subsequent attacks is at least as old as Thucydides and probably dates from the formation of relatively permanent social groups. The mechanism of this resistance is in many cases still quite unclear and, even when one of the factors at work is

known, the importance of others has usually not been determined.

For most of the twentieth century acquired resistance has been ascribed to the formation by the host of antibodies directed against the microbe. Yet the proven effects of antibody on micro-organisms are relatively few (see Section III. C of the Introduction) and, while they can account for immunity to e.g. pneumococcal infection or tetanus, many infectious diseases remain, in which the presence of antibody does not correlate with immunity or in which the parasite is not known to be affected by antibody.

The concept of cellular immunity is an old one, and the controversy between the proponents of the cellular and humoral theories of immunity, a heated one in the early years of this century, was provisionally resolved in favour of those who attributed greater importance to antibody. Nevertheless, in a number of diseases, notably tuberculosis, the antibody response is not a marked feature and appears unrelated to the outcome of the disease. Intracellular bacteriostasis of unknown mechanism seems to play the major role. In agammaglobulinaemia, again, there is strong evidence for non-humoral factors in resistance to disease. Transplantation immunity too provides an example of an immunological response readily transferred to a suitable recipient by lymphoid cells from a sensitized donor, and transferred with difficulty, if at all, by serum.

Tuberculosis is the most prominent of the bacterial

diseases in which, because of failure to relate acquired resistance to serum factors, mechanisms of cellular immunity have been repeatedly invoked. Certain other diseases, e.g. brucellosis, tularaemia, glanders, typhoid fever, chancroid, share a number of features with tuberculosis:

- the infection tends to chronicity and latency;
- acquired resistance is slow to develop and not clear-cut;
- delayed-type hypersensitivity is conspicuous in the immune response;
- several, and perhaps all, of the causative organisms are facultative intracellular parasites.

Delayed-type hypersensitivity is classically manifested in the tuberculin reaction. Despite intensive work since its discovery by Koch, the biological role of this form of reactivity and its relation to acquired resistance in this disease are still quite unclear. Indeed investigations of the nature of these manifestations have remained relatively unprofitable. The reasons for this are in part technical and in part related to the characteristics of the immune reaction in tuberculosis. Thus acquired resistance is tenuous and a steady state may be set up, in which the bacteria persist without multiplication and apparently unattacked (Rees and Hart, 1961). This probably indicates that the immune mechanism is not bactericidal. A challenge infection is free to initiate lesions and multiply as readily in BCG-vaccinated mice as in normal mice (Lévy et al., 1961), the increased resistance of the vaccinated animals

manifesting itself at a later stage. The generation-time of the organism is long, both *in vivo* and *in vitro*, a hindrance to all procedures, especially tissue-culture methods.

In other infections of this group the activity of antibodies must be taken into account, e.g. as opsonins in brucellosis, and investigation of tularaemia or glanders is limited by the risks inherent in working with these organisms.

Listeriosis in mice was found (Mackaness, 1962) to provide an experimental model with many desirable features. The organism, a facultative intracellular parasite, is pathogenic for a wide range of hosts. The clinical course of the disease is acute; convalescent animals display an acquired resistance which is bactericidal and highly efficient and in which serum factors play no role. Delayed-type hypersensitivity is readily demonstrated in convalescent mice.

It seemed therefore that this experimental system offered considerable advantages for the investigation of the nature of delayed-type hypersensitivity and its relation to acquired resistance in a bacterial disease analogous in important respects to tuberculosis.

# DELAYED-TYPE HYPERSENSITIVITY

## II. A. Historical Background and Definitions

The first observation of what is now known as delayed-type hypersensitivity was probably the "immune" or accelerated reaction that Jenner noted on variolation of a person previously infected with cowpox (Jenner, 1798). He commented also on its persistence: "It seemed as if a change which endures thro life had been produced...". In 1891 Koch described his eponymous phenomenon and subsequently reported a similar reaction to tuberculin (Koch, 1891a). Their immunological basis was not recognised clearly until the concept of allergy was put forward by von Pirquet (1911). Zinsser (1921) pointed out the delayed nature of the cutaneous reaction to tuberculin in tuberculous animals as opposed to the early responses elicited in animals anaphylactically sensitized. Soon after Opie (1924) had shown the relation between early skin reactions and serum antibody, Zinsser and Mueller (1925), failing to transfer tuberculin sensitivity with serum, stressed the possibility that this reaction was due to some cell-associated factor.

Throughout this dissertation, the term "delayed-type hypersensitivity" is used to describe the state especially notable for the occurrence of delayed skin reactions. "Delayed sensitivity" and "delayed reactivity" are often used for the sake of brevity, although strictly incorrect, the state being constantly present and only its manifestations delayed. The term "Arthus hypersensitivity" describes the state in which

the intradermal injection of antigen calls forth a reaction maximal at 3 to 6 hours and declining rapidly thereafter. Such "Arthus hypersensitivity" is transferrable to a normal animal by the injection of serum from a reactive animal.

Gell and Benacerraf (1961) offer as a working definition of a pure "delayed" reaction: "an immunologically specific inflammatory reaction which takes some hours to reach a maximum, occurring in the absence of demonstrable antibody of the conventional type". Other usual characteristics of the reaction are that it is erythematous and/or indurated, that it is transferable by cells and not by serum and that it is diminished by short-term treatment with corticosteroids and little affected by antihistamines. It may be difficult to differentiate this reaction from a "combined" reaction in animals possessing both Arthus and delayed-type hypersensitivity.

## II. B. Occurrence of Delayed-type Hypersensitivity

Reactions of delayed-type have been demonstrated in man, rabbit and guinea-pig (Raffel, 1954) and, though elicited with more difficulty, in chickens (Szenberg and Warner, 1962), in mice (Fenner, 1948; Gray and Jennings, 1955) and in rats (Flax and Waksman, 1962). The homograft reaction, which has features in common with delayed-type hypersensitivity (Medawar, 1959), occurs also in fish (Hildemann, 1962). In man and the guinea-pig the capacity to become thus sensitized is very highly developed and in the contact sensitivities of

guinea-pigs has been shown to be partly under genetic control (Chase, 1959).

Reactivity of "tuberculin" type is reported as a consequence of infection by, or immunization with, parasites of many types (Chase, 1956; Lawrence, 1956; Andrews, 1962):

| | | |
|---|---|---|
| bacteria | - | Brucella spp., Strep. pneumoniae, T. pallidum, Salm. typhi, B. tularense |
| viruses | - | vaccinia, mumps, mousepox, lymphogranuloma venereum |
| fungi | - | Coccidioides immitis, H. capsulatum, Trichophyton spp. |
| protozoa | - | Leishmania donovani, Toxoplasma gondii |
| helminths | - | Echinococcus granulosus, T. suis. |

It is well established that a hypersensitivity of delayed-type may occur on contact with certain plants such as poison ivy or sumac, with a number of chemicals, e.g. picryl chloride and 2,4-dichlorobenzene (Chase et al., 1955), or even with metallic ions (nickel and mercury).

In the recipient of an allogeneic skin graft, intradermal injection of the donor's cells produces a reaction "outwardly and histologically" similar to a tuberculin reaction (Brent et al., 1958, 1962). Delayed-type hypersensitivity is also reported as a result of insect bites and in guinea-pigs a similar reaction is stated to occur naturally (i.e. without previous sensitization) on injection of serum proteins from other guinea-pigs (v. infra).

In evaluating such reports one must consider the following features:

the description of the skin reaction, especially its appearance at 3-6 hours, the time at which it is maximal and its duration;

the occurrence of ocular and systemic reactions;

the method of sensitization, if experimentally induced;

whether the reaction can be transferred by serum, by cells;

the possible presence of toxins in the test material.

If these criteria are applied to the published descriptions, the following infections furnish examples of reactions which are in essential respects closely similar to a tuberculin reaction (the Platonic idea of this response):

diptheria (Pappenheimer and Lawrence, 1948a-c), haemolytic streptococcal infections (Mackenzie and Hanger, 1927; Lawrence, 1952), tularaemia (Foshay, 1932), brucellosis (Benedict and Elberg, 1953), H. ducreyi infection (Kornbluth et al., 1941), mumps (Enders, Cohen and Kane, 1945), lymphogranuloma venereum (Frei, 1925, 1927), vaccinia (Hooker, 1929), cryptococcosis (Salvin and Smith, 1960), coccidiomycosis (Smith et al., 1948).

This list is not intended to be exhaustive and other undoubted examples of delayed-type hypersensitivity in microbial infections could be quoted, nor is it implied that in each of the cases cited all the possible criteria have been fulfilled. It is important to emphasize that for some diseases the published reports are inadequate or conflicting (e.g. syphilis: Noguchi, 1911; Rich et al., 1933; typhoid: Gay

Force, 1913 ; T. solium infestation: Coventry, 1929).

In the conditions listed above, where the occurrence of delayed-type hypersensitivity seems well established, this state has arisen as a result of natural or experimental infection. The cutaneous reaction reaches a peak at 24-48 hours and may take a further 3-7 days to fade. Reactions evolve more slowly in large animals than in small (Gell and Benacerraf, 1961). The antigen used for testing may be soluble, as in tuberculosis, diphtheria and haemolytic streptococcal infections, a preparation of killed organisms, as in most of the virus infections, tularaemia and sometimes in brucellosis, or an extract of the organism, e.g. brucellergin; the properties of the preparation will determine in part the persistance of the antigen at the reaction-site and thus may affect the rate of evolution of the reaction.

It is instructive to examine in some detail the evidence for the occurrence of delayed-type hypersensitivity in the much studied pneumococcal infections. The toxic nature of the pneumococcal extracts often used complicates assessment. The reactions dealt with by Julianelle (1930a-d) almost certainly included a considerable Arthus component (he produced sensitization by the intradermal injection of killed organisms), though the occurrence of ocular reactions probably indicates the presence of delayed-type hypersensitivity as well (see Section II. C). Bull and McKee (1929) report a skin reaction of delayed-type to pneumococcal autolysates,

manifested as early as 3 days after infection, i.e. 3-4 days before antibody was detected. The "delayed" reactions investigated by Harley (1935, 1937) and Angevine (1941) are inadequately described.

All of this work was done in the rabbit, an animal in which Arthus sensitivity finds much more extreme expression than does delayed-type hypersensitivity. The picture is further clouded by the occurrence in pneumococcal infections of the C-reactive protein (review by Hedland, 1961); if this protein is present, injection of pneumococcal preparations containing somatic polysaccharide calls forth an "intermediate" reaction - maximal at 6-10 hours and ebbing after 18 hours (Francis and Abernathy, 1934).

In clinical cases of pneumonia (7 only) Tillett and Francis (1929) found early reactions to polysaccharide and reactions very like delayed-type hypersensitivity to pneumococcal protein, whereas Bigelow's descriptions (Bigelow, 1922) of skin reactions to similar materials, though inadequate, suggest the reverse. Herrold and Traut (1927) elicited skin responses in controls, but not in cases of pneumococcal infection (cf. the Schick test). They state: "It may be concluded, at least tentatively, that the gonococcus, meningococcus, typhoid bacillus.....when grown in broth produce substances which act on the skin in such a manner as to merit further study." This conclusion, now as then, is unassailable.

A number of reports claim "delayed" sensitivity to

insect bites, e.g. Mellanby (1946) and Hudson, Feingold and Kartman (1960); the latter authors liken the reaction to the Jones-Mote phenomenon, which follows the injection of minimal amounts of proteins and which is "delayed" probably because antibody, likewise present in minute concentration, is slow to accumulate in sufficient quantity at the injection site (Jones and Mote, 1934; see Section II. E). In both reports it is clear that the response changes over a period of a few weeks or months, in a fashion unlike delayed-type hypersensitivity, and the activity of toxins has not been taken into account.

Another phenomenon, the status of which is still more dubious, is the naturally occurring delayed reaction which Battisto (1960) detected in guinea-pigs: a reaction temporally but not morphologically similar to delayed-type hypersensitivity, and which may well prove to be connected with allotypy of serum proteins.

Nature of the antigen

Material which will elicit a delayed-type hypersensitivity reaction is for convenience referred to as "antigen" although for the most part the "antigen" by itself will not induce the state of delayed-type hypersensitivity (see Section II. E). In most bacterial hypersensitivities the constituent active in the reaction is derived either from the medium in which the organism grows or from the organism itself and is protein in nature, at least this is so in the case of tuberculosis, diphtheria, brucellosis and haemolytic streptococcal infections (Raffel, 1954). The polysaccharide

of tuberculin is inactive (Long, 1958). However, in certain fungal infections, histoplasmosis and coccidiomycosis, there is good evidence that the active component of the test reagent is polysaccharide (Edwards et al., 1963; Knight et al., 1960; Salvin, 1963). Choucroun's group (Choucroun et al., 1960, 1958; Choucroun, 1947) state that the lipopolysaccharide "PmKo" of tubercle bacilli elicits cutaneous reactions of delayed type in tuberculous patients, but this reaction, while of delayed onset, may last for some weeks and its exact nature is at present unclear.

It is obvious that the materials used in skin-testing are complex. The number of antigenically distinct proteins in tuberculin is unknown but probably large (Boyden and Sorkin, 1956). Seibert et al. (1955) showed that spme protein fractions of tuberculin possessed less activity than others in the skin test, compared on a weight basis. The proteins were all electrophoretically inhomogeneous, with one major and several minor components. Rhodes (1961) concluded that most of the subfractions of a tuberculin preparation could elicit cutaneous reactions, though the results of the immunodiffusion investigations presented do not exclude the view that one or two proteins present in varying amounts are responsible. As tubercle bacilli produce delayed hypersensitivity to many soluble proteins injected in a suitable vehicle (see Section II. E), it seems that in tuberculosis at least all proteins should be active. In this as in other respects tuberculosis may be exceptional; Benedict and Elberg (1953a) separated from

Brucella suis two protein fractions, one of which was active in the cutaneous test, the other not, though it stimulated antibody formation.

What has just been said will explain why it has not been possible in bacterial hypersensitivities to determine the degree of specificity of the reactions (the extreme precision of serological reactions has been known since the work of Landsteiner). It is known, for instance, that purified protein derivative (PPD) from cultures of avian tubercle bacilli gives larger reactions in animals infected with these organisms than in animals infected with M. tuberculosis var hominis. However PPD from cultures of the latter organism elicits reactions even in animals immunized with M. phlei (Boyden and Sorkin, 1956). Studies on the delayed-type hypersensitivity induced by adjuvants (see Section II. E) to purified proteins show that in this system the degree of specificity approaches that of conventional antibody, but that, if protein-hapten conjugates are used, the specificity is directed against the protein moiety and not the hapten (Gell and Benacerraf, 1961).

## II. C. In Vivo Manifestations of Delayed-type Hypersensitivity

The various ways in which a state of delayed-type hypersensitivity expresses itself will be exemplified largely from studies on tuberculosis. While tuberculin hypersensitivity is one of the more extreme forms of bacterial allergy, it is also by far the best studied.[1]

Local Reactions. Whether the manner of sensitization has been such that an early (Arthus) or a delayed reaction will be the outcome, directly upon injection of the eliciting dose of antigen there follows a phase, the "immediate reaction", of increased capillary permeability. This is due to the release of histamine and its duration is about one hour.

The Cutaneous Tuberculin Reaction. When tuberculoprotein (e.g. 5µg in 0.1 ml.) is injected into the skin of a tuberculous guinea-pig, little appears to happen for several hours, the immediate reaction being undetectable visually in this animal. Measurement shows that after a few hours the skin begins to thicken, and after six to eight hours the injection site is surrounded by erythema, while induration is palpable. Both erythema and thickening increase until the reaction reaches a maximum at 48-72 hours. The swelling is firm, unlike the softer swelling of an Arthus reaction. In severe reactions in the guinea-pig, but not necessarily in other species, necrosis often occurs.

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[1] Descriptions are based on those of Boyden (1958) in the main.

Histologically the reaction is mainly cellular, mononuclear cells and granulocytes appearing in increased numbers as early as four hours. At 24 hours the predominant cell is the large mononuclear, characteristically found in perivascular accumulations, and lymphocytes are also present.

The Koch Phenomenon. In a normal guinea-pig subcutaneous injection of virulent tubercle bacilli produces a nodule which breaks down to form a persistent ulcer. The infection is disseminated to the regional nodes and beyond. In a tuberculous animal, the same procedure yields an area of inflammation which ulcerates superficially and then heals, the regional nodes usually remaining uninvolved.

The Corneal Reaction. If tuberculin is applied to the scarified cornea or injected into it, the ensuing reaction is closely similar to the cutaneous reaction, both in timing and in histology. Considerable stress is often laid on the corneal reaction as a manifestation peculiar to delayed-type hypersensitivity, but this question is not settled. Waksman and Bullington (1956) failed to produce a response in the eyes of rabbits possessing passive Arthus sensitivity by intracorneal injection of the antigen. However the work of Julianelle (1934) and of Köllner (1913) does not agree with theirs. Corneal cells from tuberculous animals show no sensitivity to tuberculin in vitro (May and Weiser, 1956).

## Systemic Reactions

Shock. If a tuberculous guinea-pig receives a

large dose of tuberculoprotein intravenously, little appears to happen immediately. After some hours the animal is obviously unwell, but displays none of the excitement characteristic of anaphylaxis. With a sufficient dose of tuberculin death occurs in 6-36 hours. At autopsy the liver, lungs and spleen show small red spots, which consist of an inflammatory reaction in the vicinity of tubercles. This "focal tuberculin reaction" occurs also on injection of sublethal doses of tuberculin (Steidl and Heise, 1931).

The reactions about to be discussed might more appropriately be described as "distant" rather than "systemic". In the guinea-pig a peritoneal exudate induced by glycogen administered four days previously contains a high proportion of macrophages. If the animal shows tuberculin sensitivity, injection of as little as 0.1 μg of PPD by subcutaneous, intraperitoneal, or intravenous routes produces a profound fall in the macrophage content of the exudate. The same effect is apparent in animals possessing a mixed Arthus and delayed-type hypersensitivity to ovalbumin, but not in animals with pure Arthus sensitivity (Nelson and Boyden, 1963). If large doses of tuberculin are injected into the peritoneal or pleural cavities, the cells harvested 24 hours later are mostly dead if the animal is tuberculous, but cells from normal animals are living (Holst, 1921; Stewart, Long and Bradley, 1926).

In mice recently recovered from listeriosis, mitoses appear in the focal lesions in spleen and liver during a challenge

infection (Mackaness, 1962). Similarly, the injection of listerin into such animals induces marked mitotic activity and proliferation on the part of the peritoneal cells (Mackaness, 1964b).

The phenomena described in the preceding two paragraphs may well be related, since a similar range of effects is reported to follow in vitro, if cells from such animals are exposed to the appropriate antigen (see Section II. D). In this context it is of interest to note that leukaemoid reactions, which are characterised by the presence of "blast" cells and other immature forms in the peripheral blood, have been found in association with tuberculosis more frequently than with any other infection (Wintrobe, 1961).

In 1959 Crowle used increase in lung density on intravenous injection of tubercle bacilli as a measure of delayed-type hypersensitivity in mice. He did not, however, present evidence that the two phenomena were related and subsequent work by Myrvik, Kawata and Leake (1962) on what is probably a similar reaction makes this unlikely. The latter group found that in the lungs of rabbits sensitized with BCG the numbers of macrophages increased sharply after intravenous administration of a further dose of BCG and that this response was not inhibited by intravenous injection of tuberculin, nor was it correlated with dermal sensitivity to tuberculin.

## II. D. In Vitro manifestations of Delayed-type hypersensitivity

In 1928 Rich and Lewis reported that leucocytes and spleen and bone-marrow cells derived from animals giving positive tuberculin reactions are hypersensitive to tuberculin *in vitro*, the presence of tuberculin in the medium leading to inhibition of migration, morphological changes and death (Rich and Lewis, 1928, 1932). Subsequently this phenomenon has been widely studied, as it appeared to offer promise, as yet unfulfilled, of an *in vitro* assay for tuberculin sensitivity. Cells derived from various species, e.g. man, guinea-pig, mouse, rabbit, and from many tissues, including lymph node, spleen, bone marrow, blood, exudates, lung, skin, kidney, liver have been examined [this work has been reviewed by Waksman (1958) and Wasserman (1962)]. Most workers have looked for effects on morphology (rounding up of cells, granulation and vacuolation), or on migration; others have considered physiological activities (respiration, glycolysis, growth, enzyme activities). Animals showing hypersensitivity to a variety of antigens, e.g. tuberculin, *Brucella* antigens, mumps virus, have been used.

A frequent finding (e.g. Dittmar and Sixel, 1954; Heilman *et al.*, 1944; Leahy and Morgan, 1952; Rich and Lewis, *op. cit.*) has been toxic changes followed by death, inhibition of migration,etc. However, failure to observe such effects is reported (e.g. Marks and James, 1953; Baldridge and Kligman, 1951). In some hands the addition of specific antigens to the culture medium results in

stimulation of the cells, e.g. increased growth of fibroblasts from tuberculous chickens (Fischer, 1928), more rapid maturation and even proliferation of peritoneal exudate cells (Waksman and Matoltsy, 1958). Peripheral blood cells from Mantoux-positive donors without active tuberculosis undergo mitosis in culture in the presence of tuberculin (Pearmain et al., 1963; Marshall and Roberts, 1963a).

The relationship of this phenomenon to delayed-type hypersensitivity is not entirely clear. It is tempting to assume that the in vitro reaction in some ways reflects the mechanism of in vivo delayed reactions. However, the concentrations of, e.g., tuberculin required to produce changes in sensitive cells are high and the difference between the concentrations active on sensitive and normal cells is slight. Rich and Lewis used 1/60 Old Tuberculin and controls reacted to 1/10, i.e. a ratio of 1:6. Heilman and Seibert (1946) found the same ratio to hold, and in brucellosis control cells responded to a concentration of antigen tenfold greater than that affecting sensitive cells (Heilman et al., 1958). Even the 1 µg/ml. of PPD shown by Wasserman (Wasserman, 1962a) to be toxic is a great deal, since 0.1 µg or less will elicit a reaction in a highly sensitive guinea-pig, while 1 mg. produces no effect in a normal animal. Here the ratio of the quantity active in sensitive animals to that active in normal animals is 1/10,000 or more. The concentration used by Pearmain et al. to induce mitosis was apparently ca 1 µg/ml. Stimulatory effects have been observed usually when smaller doses are used.

The time-scale of the reactions is not always similar to that of delayed-type hypersensitivity. Toxic changes occur over 24 or 48 hours. Proliferative changes develop later, 48-72 hours in the system used by Waksman and Matoltsy (1958), while 4-6 days are required for the onset of mitotic activity in peripheral blood cells (Pearmain et al., 1963).

The specificity of the reactions described may in many cases be questioned, since often controls have not included cultures containing unrelated proteins toxic for normal cells in the concentrations used. Moen and Swift (1936) state, without presenting details, that the toxic materials used, e.g. streptococcal autolysates, had "little, if any, greater effect on tuberculin sensitive cells". Wasserman, drawing his cells from animals infected either with BCG or with mumps virus, was able to show a specific effect of mumps antigen on mumps-sensitized cells (Wasserman, 1962a). Similar results were obtained by Darlington and Scherago (1960) and Heilman et al. (1962), using brucellergen and tuberculin.

The methods most often used are insensitive and subject to numerous errors (v. Boyden, 1958) and few workers have submitted their results to statistical analysis (Packalen's and Heilman's groups are exceptions, cf. Wasserman, 1962; Heilman et al., 1960).

Despite these objections, it seems very likely that macrophages, and to a lesser extent fibroblasts, derived from

animals possessing delayed sensitivity to an antigen show altered behaviour in tissue culture when in the presence of the antigen. The apparently opposed effects noted, ranging from death to mitosis, are probably explained by differences in the following variables (among others):

(a) Nature of the sensitizing programme (e.g. live or killed organisms, number of doses, etc.); this determines the level of hypersensitivity induced.

(b) Time elapsed between skin testing and tissue culture investigation - relevant because of the possibility of desensitization.

(c) Dose of antigen used in the culture.

Cells of epithelial origin have not usually been found to be affected. With granulocytes and lymphocytes the position is unclear, despite some positive reports (Heilman et al., 1958, 1960; Wesslén, 1952) because of possible confusion with the phenomenon of lympholysis (v. infra.). Heilman et al. (1958, 1960) state that the "leucocytes" of splenic tissue are less sensitive than the macrophages. If the analogy with the effects of phytohaemagglutinin is valid, then the cells stimulated by tuberculin to divide in culture are probably small lymphocytes (Marshall and Roberts, 1963). Mitoses may appear in such cultures, however, in the presence of a soluble antigen such as tetanus toxoid, if the donor has been immunized with toxoid (Elves et al., 1963).

Lympholysis

The susceptibility to tuberculin of leucocytes from tuberculous animals was reported by Holst in 1922 and by Long and Stewart in 1926. In 1947 Favour described lysis of lymphocytes derived from tuberculous guinea-pigs and rabbits when incubated *in vitro* in the presence of tuberculin. Lysis was complete in a few minutes to an hour. In mice lymphocytes only were affected, in guinea-pigs and man granulocytes and macrophages were lysed subsequently (Favour, 1951). This finding has been confirmed (e.g. Dittmar and Sixel, 1954; Wesslén, 1952; Waksman, 1953 ), but the subsequent report (Miller and Favour, 1951), to the effect that the factor responsible for lysis was present in the sera of the animals and that it was released on incubation of this serum with tuberculin, is controversial (Wesslén, 1952; Janicki, 1959). Waksman (1953) agreed that complement was necessary for lysis and could find no correlation between lympholysis and cutaneous hypersensitivity, although the latter was related to inhibition *in vitro*.

There are two objections to this work. If cells from animals endowed with Arthus sensitivity to an antigen are incubated with that antigen, agglutination and hence an apparent fall in the cell-count may occur, along with toxic changes (Hartman and Hoch, 1955). Waksman (1953) found lysis in a similar system. The granulocytes seem however to be the main cell involved. Furthermore, in most of these studies, the only evidence of cell-lysis is a fall in the differential count;

the possibility is thus neglected that clumping, adhesion of cells to the walls of the tube or even autophagocytosis may have occurred. Hence this phenomenon, if it occurs, is probably unrelated to delayed-type hypersensitivity.

## II. E. Factors in the Induction of Delayed-type Hypersensitivity

It was observed early that tuberculin, which elicits a delayed reaction in infected subjects, on injection into normal animals calls forth simply the production of antibodies and Arthus hypersensitivity (Baldwin, 1910). And in general it has been found that soluble antigens injected in aqueous solution by whatever route do not produce delayed-type hypersensitivity; the Jones-Mote phenomenon is neglected here, as there are reasons for setting it apart from the former reactivity (Crowle, 1962). The intact bacterial cell has usually been found to be essential, unless special vehicles are used (and v. infra). Killed bacteria are effective in some cases, e.g. mycobacteria (Petroff and Stewart, 1925) and perhaps pneumococci (Julianelle, 1930d; but see previous discussion, Section II. B.). The administration of killed tubercle bacilli in saline leads to a rather low level of delayed-type hypersensitivity and more antibody than does natural infection. In brucellosis only the live organism induces hypersensitivity (Spink, 1956).

For experimental purposes, the use of living organisms has certain disadvantages, as the effect of the dose depends not only on the number of viable units injected but also on

their physiological state and the extent to which the natural resistance of the host allows them to multiply before the infection is controlled. Materials used to detect the hypersensitivity are complex mixtures, which have proved difficult to purify (Seibert, 1941, 1950) and it was therefore desirable to produce delayed sensitivity to simple proteins.

This was first achieved in Dienes' laboratory (Dienes and Schoenheit, 1927). Ovalbumin was injected into tuberculous foci of guinea-pigs and a subsequent skin-test with ovalbumin elicited a reaction of delayed type, sometimes free of an Arthus component, though the results achieved were very variable (Dienes, 1928, 1929). As a result of the work of Freund's group (reviewed by Freund, 1956) the use of "complete" adjuvants was introduced. Incorporation of an antigen in mineral oil and a suspending agent ("incomplete adjuvant") leads to abundant and prolonged antibody production from a single dose. The addition of heat-killed acid-fast bacilli to the incomplete adjuvant results in delayed-type hypersensitivity to the protein, but there is an associated antibody response and a considerable Arthus component in the skin reaction. A wax fraction of the tubercle bacillus is apparently the active agent (Raffel, 1948, 1950; Raffel *et al*, 1949; Myrvik and Weiser, 1952). However, while the combination of wax and tuberculo-protein induced a sensitivity virtually indistinguishable from that of tuberculosis, skin tests with ovalbumin in animals receiving ovalbumin and wax yielded reactions by no means so typically "delayed". There

is as yet no evidence that a wax with these properties is present in all microbes, infection with which results in delayed-type hypersensitivity.

More recently other procedures have been introduced. Uhr, Salvin and Pappenheimer (1957) used antigen-antibody complexes in incomplete adjuvant, Gell and Benacerraf (1959) advocated denaturation of the protein and Benacerraf and Gell (1959) conjugation of the protein with haptens. The state produced by these methods is probably more akin to Jones-Mote hypersensitivity (Raffel and Newel, 1958; Nelson and Boyden, 1963).

The specific effect of tubercle bacilli on the course of sensitization, mediated presumably by the wax contained therein, may be considered to represent an inductive factor contributed by the parasite. Certain aspects of the host response have also been held to play a major role in the inductive process. "The route of sensitization appears to condition the type (of) sensitivity which is induced in the host" (Lawrence, 1956). Thus Swift's group (Andrewes, Derick and Swift, 1926; Derrick and Swift, 1929; Swift and Derrick, 1929; McEwen and Swift, 1934), working with an "indifferent" streptococcus, found that intradermal injection of the organism produced delayed-type hypersensitivity, whereas intravenous injection was ineffective, leading only to "immunity" (apparently lack of reactivity on skin-testing is meant). The results of Julianelle (1930) are in agreement with these. However Debré, Paraf and Dautreband (1920) showed that induction of

hypersensitivity by BCG simply took longer if the dose was injected intravenously instead of subcutaneously. Clawson (1935) found living BCG equally effective by either route. In view of the widespread occurrence of delayed-type hypersensitivity after infection, it would appear unjustified to stress the importance of the skin in its induction (contact sensitivity always excepted, Chase, 1959).

Focal granulomatous lesions are characteristic of the infections notable for delayed-type hypersensitivity. It has often been suggested (Dienes, 1930; Hanks, 1935; Freund, 1947; Burnet and Fenner, 1949) that the type of sensitization, i.e. early or delayed, is conditioned by the cellular response of the host to the invading microbe. The work of Dienes on the induction of hypersensitivity to ovalbumin by injection into tuberculous foci and the effects of mycobacterial wax, the histological response to which is very like that to mycobacteria (White, Coons and Connolly, 1955), are observations usually adduced in support of this view. Boyden (1958) suggests that the importance of the cellular reaction may lie in the liberation of antigens within a particular type of cell, the macrophage, thus in a situation where they may well be modified before reaching the immunologically competent cells. While these speculations are attractive, evaluation of the relative contributions of parasite and host to the determination of the sensitivity induced must await a clearer understanding of the nature of the delayed reaction.

## II. F. Modification of the Delayed-type Hypersensitivity Response

Under this heading are discussed factors which may inhibit, partly or completely, the development of delayed-type hypersensitivity under conditions of otherwise adequate stimulation or which may decrease or abolish the expression of an already established state. Such factors in the response as the dose of infecting organisms and the route by which they gain entry will be treated later.

### Immunological Tolerance

Both rejection of an allogeneic skin graft and the production of serum antibody can be prevented if the animal encounters the relevant antigens before immunological maturity. On the basis largely of Owen's work with cattle chimaeras (Owen, 1945) and Traub's observations on lymphocytic choriomeningitis virus (Traub, 1938), Burnet and Fenner suggested in 1949 that, if in embryonic life cells from a genetically distinct race were implanted and established, no antibody response would develop against the foreign cells after birth. The validity of this concept was subsequently demonstrated by Medawar's group (Billingham, Brent and Medawar, 1953) with respect to tissue grafts and injection of heterologous protein was soon shown likewise to prevent an antibody response to subsequent doses of the protein (Hanan and Oyama, 1954).

However, attempts to induce this immunological tolerance to bacterial antigens have yielded equivocal results.

Burnet's group (Burnet, Stone and Edney, 1950), Cohn (1957) and Nossal (1957) reported failure with a variety of bacterial and viral antigens. In birds infected with avian tumour viruses tolerance seems to become established, as antibody production is absent (Rubin, 1962). As with lymphocytic choriomeningitis virus, infection must occur during embryonic life. Buxton (1954) achieved some depression of the antibody response to infection by the injection of killed Salm. pullorum into chick embryos, but the results are not clear-cut and the work is technically somewhat unsatisfactory (Cohn, 1957). Similar criticisms can be levelled at the results of Lindorfer and Subramanyan (1959) and of Friedman and Gaby (1960), who used staphylococcal toxoid and killed Sh. paradysenteriae respectively.

Attempts to inhibit the induction of delayed-type hypersensitivity by such procedures have been similarly successful. Weiss and Wells (1957) and Weiss (1958) injected guinea-pigs in utero with Old Tuberculin, killed and living BCG and found that in some cases the response to subsequent injections of killed BCG was depressed or absent. Milgrom, Wicher and Ragala (1958) injected living BCG into guinea-pigs in utero and reported that the nine survivors did not react to tuberculin, whereas they resisted a subsequent challenge better than the five (probably unsuitable) controls. However, in the similar experiments on mice of Rees and Garbutt (1961) the animals showed evidence both of immunity and of delayed-type hypersensitivity.

In most of these experiments, if non-replicating antigens were used, injections were not repeated at intervals after birth, a procedure shown to be necessary in rabbits rendered immunologically tolerant to proteins (Smith, 1961).

In summary it is fair to say that, with the exception of the two viruses mentioned, immunological tolerance to microbial antigens has not been clearly demonstrated.

Effect of Prior Treatment with Antigen in Adult Life

In evaluating this work account must be taken of another phenomenon, described by Boyden (1957). It was known from the experiments of Barr and Llewellyn Jones (1953, 1955) that the response to one antigen may be depressed by injecting it together with a second antigen to which the animal is previously sensitized. Boyden injected guinea-pigs with an unheated tuberculoprotein preparation known to produce antibody, and subsequently with BCG vaccine. The level of delayed-type hypersensitivity in these animals was significantly depressed 11 days after vaccination, but equal to that of the controls later.

The mechanism of this effect is unclear. Antibody passively acquired will depress the response to the specific antigen in both guinea-pigs and man if present in adequate amounts (Barr, Glenny and Randall, 1950; Mason, Robinson and Christensen, 1955). In such circumstances it is likely that some immune elimination will take place and, in a situation where delayed-type hypersensitivity to bacterial antigens is being induced in the presence of specific antibodies, it is

possible that these antigens are eliminated partially before sensitization occurs, so that the effective dose is smaller. Alternatively, some form of antigenic competition may be occurring, as originally suggested by Boyden (1957).

The phenomenon invites comparison with the enhancement of tumour and skin homografts produced either actively by injection of tissue extracts or passively by transfer of antiserum (Kaliss, 1958; Brent and Medawar, 1962). Similarly,in experimental allergic encephalomyelitis injection of encephalitogenic extracts before their administration in complete adjuvant prevents the development of the disease (Shaw et al., 1960) and this protection is passively conferred on other animals by antibodies to brain tissue (Paterson and Harwin, 1963).

Effect of Repeated Skin-Tests

Once a state of delayed-type hypersensitivity is established, further injections of soluble antigen may affect its level in different ways. When a tuberculous infection becomes arrested, hypersensitivity gradually wanes after a time, both in experimental and clinical situations (Rich, 1951), and hence its maintenance at a high level was thought to require continual exposure. However, in 1955 Magnus and Edwards reported that a small group of BCG-vaccinated subjects who had not been skin-tested in the 3-4 years since vaccination showed significantly smaller reactions (e.g. 12.0 mm. mean induration) than others tested at yearly intervals (18.6 mm. mean induration). Magnus (1957) and Magnusson, Jespersen and

Weis Bentzon (1960) have shown the same effect experimentally in guinea-pigs. It thus seems that injection of small amounts of antigen at regular intervals, as in skin-testing, will maintain reactivity at a significantly higher level for considerable periods.

Desensitization

If the dose of antigen is larger and the interval between its injection and a subsequent skin-test shorter, desensitization ensues, a phenomenon first reported by Bauer in 1909. When sufficient quantities of tuberculin are administered at appropriate times, subsequent intra-dermal injection of tuberculin fails to elicit a reaction even in highly sensitized subjects. A single dose of tuberculin injected intravenously will desensitize for some days, the ratio of the intravenous dose to the skin-test dose being ca 10-50:1 (Pepys, 1955; Arima et al., 1962). "Permanent" desensitization is much more difficult to achieve and heroic dosage schedules, e.g. 1-2G of Old Tuberculin per day, have been employed (Rothschild, Friedenwald and Bernstein, 1934). (Much of this work will be discussed in a later section). It is likely that the "anergy" or failure to react to tuberculin often observed in the late stages of human tuberculosis has a similar basis.

Effect of Drugs and Radiation and other Factors.

The results of the injection of antigen are presumably specific, though the question has not been examined.

Various other agents are known to modify a state of delayed-type hypersensitivity in non-specific fashion. X-irradiation or treatment with drugs such as 6-mercaptopurine and methotrexate have been found to suppress the primary antibody response (Taliaferro and Taliaferro, 1951; Schwartz, Stack and Dameshek, 1958; Friedman, Buckler and Baron, 1961; Dixon, Talmage and Maurer, 1952). These same agents also inhibit, at least partially, the initiation of delayed-type hypersensitivity; this response is however more resistant than antibody production both to X-rays and to methotrexate (Uhr and Scharff, 1960; Friedman et al., op. cit.). To achieve complete suppression of delayed-type hypersensitivity in vaccinia infection, Friedman, Baron et.al.(1962) adopted a combined treatment with methotrexate and X-irradiation. Hoyer et al. (1962) showed that in doses close to lethal 6-mercaptopurine had some effect on the sensitization of guinea-pigs by BCG.

A state of hypersensitivity once established, short-term treatment with X-irradiation, nitrogen mustards or steroids will decrease or inhibit elicitation of the reaction (Pepys, 1955; Packalén, 1952; Long and Spensley, 1954; Harris and Harris, 1950). All these agents produce a fall in the numbers of circulating leucocytes.

Epsilon-amino-caproic acid diminishes the cutaneous response to tuberculin (Mitsubishi, 1957) and its derivative epsilon-acetamido-caproic acid inhibits the rejection of homografts (Bertelli and Frontino, 1963).

Cutaneous hypersensitivity reactions may be affected by local factors. In pregnancy, hypothyroidism and cachexia depression of reactivity is thought to be due to the increased rate of lymph drainage in the skin and the inhibitory effect of locally injected histamine or hyaluronidase probably has a similar basis (Pepys, 1955). Injection of adrenaline or the presence of local vascular stasis intensifies the reaction. The mechanism of inhibition in such conditions as measles, influenza and other acute infections is undetermined.

It is usually stated that anti-histamines do not alter delayed-type hypersensitivity reactions (e.g. Gell and Benacerraf, 1961; Boquet, 1943). Nevertheless reports are not lacking that depression of the tuberculin reaction can be achieved with large doses of such drugs (Pepys, 1955) and even with therapeutic levels (Graub and Barrist, 1950). In concentrations equivalent to those usually attained in treatment, antihistamines have a vasoconstrictor action (Haley and Harris, 1949), possibly by blocking the effects of endogenous histamine, so that an effect on the delayed cutaneous reaction is not unexpected.

II. G. Mechanism of the Reaction

Transfer of Delayed-type Hypersensitivity by Cells

The process whereby the injection of tuberculin into a suitably sensitized animal elicits within 24-48 hours a considerable inflammatory reaction is not understood. We have already noted that the swelling is due largely to cellular infiltration rather than oedema. It was realized early that tuberculin sensitivity was apparently unrelated to serum antibody (Baldwin, 1904). Zinsser and Mueller (1925) were in the main unsuccessful in their attempts to transfer this form of reactivity with serum, whereas this had been accomplished as early as 1909 by the use of whole blood or lymphoid tissue (Helmhoiz, 1909; Bail, 1910). However results were inconsistentand the possibility remained controversial until Landsteiner and Chase in 1942 elicited a contact sensitivity reaction in animals given peritoneal cells from suitable donors. Chase (1946) used the same technique to transfer tuberculin sensitivity and went on to show that active cells could also be derived from the "leucocyte cream" of the peripheral blood, the spleen and lymph-nodes (Chase, 1951). This work has been repeatedly confirmed (e.g. Stavitsky, 1948; Metaxas and Metaxas-Buehler, 1948; Wesslén, 1952a) and similar results have been obtained with respect to brucellosis (Metaxas-Buehler, 1951).

Since antibody production can be transferred with cells from the same sources (Chase, 1951; Harris et al. 1954, 1954 a), the important distinction in this regard between delayed-type and anaphylactic hypersensitivity is that the former can rarely (see below) be transferred with serum, whereas the latter reactivity is easily so induced in the recipient.

In most of the studies where generalized reactivity is transferred, it has been demonstrable for only a few days (Kirchheimer and Weiser, 1947; Metaxas and Metaxas-Buehler, 1955). The latter authors report, in agreement with Chase (1959), that in a proportion of animals there may be a further phase of more enduring hypersensitivity a few days after its initial subsidence, the result perhaps of active sensitization.

In inbred guinea-pigs Bauer and Stone (1961) find a rather different situation. Fewer cells are needed and sensitivity, first detectable after 6 to 8 days, increases to reach a peak in the third week and then declines. This suggests that in outbred animals passive sensitization is terminated by a homograft reaction, to which mechanism cessation of antibody production by transferred cells is very likely due (Harris, Harris and Farber, 1954, 1958).

Lymph-node cells and peritoneal exudates are always effective, spleen cells sometimes not so (Waksman and Matoltsy, 1958). Thoracic duct lymphocytes were used successfully by Wesslén (1952a), while exudates containing mainly polymorphonuclear cells are inactive (Kirchheimer, Hess and

Speirs, 1951). Chase (1951) found that the capacity of cell-mixtures to induce sensitivity paralleled their lymphocyte content, a further instance of the immunological virtuosity of a cell which Gowans has shown (Gowans, McGregor, Cowen and Ford, 1962) to restore the ability of rats to reject a skin-graft and to form antibody.

Metaxas and Metaxas-Buehler (1955) showed that there was no latent period; in animals given cells from hypersensitive donors and skin-tested at the same time tuberculin sensitivity was evident 24 hours afterwards and reactions elicited later evolved according to the same time-scale.

Recently a number of attempts have been made to delineate more exactly the function of the transferred cells. Cells from hypersensitive donors are labelled *in vivo* with a radioactive tracer, usually tritiated thymidine, the recipients are then skin-tested and the test site is examined histologically and by autoradiography. Najarian and Feldman (1961) reported a considerable excess of labelled cells at the specific reaction-site under these conditions, but their results are criticised by McCluskey, Benacerraf and McCluskey (1963), who point out that the control cell-suspensions used by Najarian and Feldman, being derived from unstimulated animals, would not contain the same types of cells. In the experiments of McCluskey *et al.* no difference was found in the numbers of labelled cells in the specific and control reactions; a conclusion with which Turk (1962) and Hamilton and Chase (1962)

are in agreement. In any event, the numbers of donor cells appearing at a test site are small, usually around 3% of the mononuclear cells; using a different technique, Turk's estimate is $10^5$ or 0.1%. But if prospective recipients receive radio-thymidine before the cell transfer, 70-90% of the mononuclears at the reaction site are labelled, just as in the actively sensitized animal (McCluskey et al., op. cit.).

If cells from the hypersensitive donor are enclosed in "Millipore" chambers and implanted into normal animals, the recipients display no cutaneous reactivity to tuberculin, whereas rejection of a skin-graft can be hastened in this fashion (Najarian and Feldman, 1962; McCluskey et al., op. cit.).

On the basis of the findings reviewed, a cutaneous reaction of delayed type may be conceived as a series of events.

1. Antigen reacts with small numbers of sensitized cells at the site of injection. Only a small minority of the cells present in the fully developed reaction are derived from the donor. These sensitized cells may be present in the skin (in actively sensitized animals) or may come thither in the blood (in actively and passively sensitized animals). The occurrence of such cells in the blood is widely attested (Chase, Stavitsky, Lawrence, op. cit.).

2. This interaction of sensitized cells and antigen induces a major influx of cells, probably by some non-

specific mechanism analogous to the release of histamine or of the chemotactic principle described by Boyden (1962). The cells thus infiltrating the region are previously "uncommitted" in the terminology used by Turk and Stone (1963), i.e. they do not embody either specific antibody against, or specific "memory" of, the test antigen. An alternative hypothesis is that on contact with the antigen the specifically sensitized cells are stimulated to produce a specific antibody which then attaches itself to the round cells of the host. It is not possible at present to decide between these two views.

This outline is intended to account for the cellular events observed. It says nothing of the way in which cells are sensitized, except to assume that, for a minority at least, the sensitization has an immunological specificity comparable to known antigen-antibody reactions.

The specific "recognition" of the antigen by these cells may be due to a cytophilic antibody such as Boyden (1963) describes or to a "high-affinity" antibody such as Karush and Eisen have envisaged (Karush and Eisen, 1962). The failure to transfer delayed-type hypersensitivity by cells enclosed in "Millipore" chambers is evidence against a mechanism involving the production of antibody by the transferred cells. (The possible role of antibody in reactions of delayed type is also discussed below).

## Transfer of Delayed-Type Hypersensitivity with Serum or Cell Fractions

In animals attempts to use subcellular fractions instead of intact cells in this type of experiment have met with no success, though Jeter, Tremaine and Seebohm (1954) elicited contact reactions in animals which had received sonicates of peritoneal cells, the same technique proving ineffective in the case of tuberculin sensitivity.

In man the situation is different. Lawrence, in an extensive series of papers reviewed by himself in 1959 and 1960, has reported that lysates of peripheral blood leucocytes from reactive donors will confer on recipients hypersensitivity to tuberculin, streptococcal proteins and coccidioidin, and the capacity to reject a skin graft in an accelerated fashion. Lawrence summarises the properties of his "transfer factor" as follows (Lawrence, 1963):

> a soluble, dialysable material of molecular weight probably less than 10,000;
>
> unaffected by deoxyribonuclease, ribonuclease or trypsin;
>
> free of albumin or globulin by immunological tests.

This work, if valid, is highly important, but it lacks general acceptance. The properties claimed for transfer factor are difficult to reconcile with what is known of antibodies or of the recognised systems of information-transfer within cells.

Reference has already been made to the general failure to transfer delayed-type hypersensitivity with serum. However sporadic reports of the achievement of this recur. In 1925 Zinsser and Mueller claimed some irregular successes. In 1955 Cole and Favour found that plasma fractions from sensitive animals induced skin reactivity to tuberculin in the recipients. Donors yielded active serum only if skin-tested in the previous 48 hours. It may be significant that the most convincing result obtained by Zinsser and Mueller was in such circumstances. It is possible that under these conditions material analogous to Lawrence's transfer factor may be present in the serum. But if serum can mediate this reactivity the precise conditions required have not yet been defined; scarcely one reaction out of sixty in the protocols presented by Rauch and Favour (1960) would appear to reach significant levels.

This is an appropriate point to refer to some recent work carried out by Boyden (1963a, 1963b). If guinea-pigs are injected with sheep red cells in complete adjuvant, a later skin-test with urea extracts of the red cells reveals a delayed-type hypersensitivity which, in many cases, is pure soon after sensitization and which never shows a marked Arthus component. When peritoneal macrophages from the treated guinea-pigs are incubated with sheep red cells, the latter adhere to them; this property of adhesiveness for a certain type of red cell can be conferred on normal macrophages by serum from the treated animals. Polymorphonuclear leucocytes

and lymphocytes are not active in this system. The cytophilic antibody involved can be eluted from macrophages in a relatively pure state and has been shown to opsonize sheep erythrocytes for engulfment by macrophages only, not by polymorphs. It will not agglutinate the red cells, but can be detected on them by the Coombs test. Animals injected with incomplete adjuvant and erythrocytes occasionally show this antibody in very low titre.

While the importance of this antibody in bacterial allergy remains to be demonstrated, it is interesting that in brucellosis Stevenson, Ferris and Lewis (1954) found a correlation between incomplete antibody and skin reactivity.

## II. H. Biological Role of Delayed-Type Hypersensitivity

Despite the intensive investigations of the last seventy years, a fraction of which has been reviewed here, there has emerged no very clear definition of the biological role of delayed-type hypersensivitity. It is natural to assume that such a widespread and complex response, and one, moreover, which apparently finds its subtlest and most marked expression in the species to which the investigators belong, has been maintained through evolutionary development by its survival value, i.e. in teleological terms, that it serves some function. One should not disregard the caveat entered here by Topley and Wilson (1955), who point out that "the only reliable estimates of survival value are those made directly on the species as it is now established in the animal world".

Discussion of the relationship of delayed-type hypersensitivity to acquired resistance will be deferred for the present. Here it is proposed to mention briefly some other aspects of the delayed response in the manifestations of disease.

If contact sensitivity to chemicals and other substances has the same mechanism as delayed sensitivity, and this seems very probable, then here the response would appear to be deleterious.

There is some evidence to implicate delayed-type hypersensitivity in certain pathological processes of infectious disease. In tuberculosis the formation of caseous tissue and of cavities is said to be due to hypersensitivity reactions, since injections of tubercle bacilli directly into the lungs of animals sensitized to this organism results in a caseous lesion which goes on to cavitation (Crowle, 1962, who reviews the work of some

Japanese investigators). Ovalbumin induces similar lesions in animals possessing delayed sensitivity to this antigen, but not in those the sensitization of which is of Arthus type.

In brucellosis delayed-type hypersensitivity is held responsible for most of the symptoms, and in fact exposure of highly sensitive persons to Brucella antigens, even in the form of an aerosol, may reproduce in them all the symptoms of an acute attack.

A characteristic manifestation of one group of infectious diseases, the acute exanthems, is a rash and delayed reactivity has often been inculpated in its production (von Pirquet, 1913). In 1948 Fenner reviewed the pathogenesis of the acute exanthems (Fenner, 1948a) and cited a good deal of evidence (derived in part from his own studies on ectromelia in mice) to show that the production of the rash is related to the multiplication of the virus in the skin. However in 1963 Flick and Pincus reported that guinea-pigs infected with vaccinia developed no primary skin lesion when treated with an anti-mononuclear-cell serum, although the infection resulted in antibody production comparable with that of the controls. These views are of course readily reconcilable; a vesicle may result from a delayed reaction to a deposit of virus arising by local multiplication in the skin.

To what extent delayed-type hypersensitivity is implicated in the causation of the auto-immune diseases (Mackay and Burnet, 1963) is not yet clear and will not be further discussed here.

These scattered observations reveal aspects of the delayed response which must figure, in one or other column, in the immunological balance-sheet.

## III. MECHANISMS OF ACQUIRED RESISTANCE

> It is plain that the microbial diseases for which there is no explanation whatever of immunity mechanisms far outnumber those where susceptibility and resistance can be explained in terms of recognised immunological reactions - cellular or humoral. R.J. Dubos (1954).

### III. A. Complex Nature of Acquired Resistance

Acquired resistance to microbial disease, while the oldest known immune phenomenon, is the resultant of the interaction of many factors, and the present state of our understanding of the processes involved is epitomized in the above quotation.

The results of immunization procedures may over-simplify the picture. That antibody artificially induced to a particular antigen protects against a microbial infection does not prove that this one constituent of the organism is alone of importance in resistance. In pneumococcal infections, for example, it is clear that the main determinant of virulence, the capsular polysaccharide, is "the essential immunizing factor" (Raffel, 1953). Certainly immunization with this type-specific substance protects against infection with organisms of the same type and in patients convalescent from pneumonia antibody to this polysaccharide appears in the serum. However there is evidence that this is not the whole story. Bull and McKee (1929) found that rabbits injected with killed pneumococci of types II, III or IV were protected completely against intranasal challenge with type I. The immunization procedure

induced low titres of type-specific anti-capsular antibody and moderately high titres of complement-fixing antibody against all three organisms. Also vaccinated animals may show the capacity to resist infection, while their serum will not protect others (Walsh and Cannon, 1936): here one must consider the anamnestic effect of the challenge.

In this disease, surface phagocytosis (Wood et al, 1946) probably plays a part, since Cohn and Morse have shown that staphylococci comparably opsonized are ingested more rapidly in vivo than in vitro (Cohn, 1962; Cohn and Morse, 1959). Moreover, pneumococcal infection may well lead to delayed sensitivity (see pp. 9-10). Thus, despite extensive investigation, the immune response to the pneumococcus presents features of unknown significance for acquired resistance.

In this context, we may not ignore what are apparently non-specific reactions, since the possibility is open that the production of such "broad-spectrum" antimicrobial factors may be triggered by more specific reactions (v. Section III.E.).

## III. B. Antimicrobial Factors of Tissues[1]

Normal tissues have yielded a number of substances which possess antibacterial activity in vitro: fatty acids, short-chain monocarboxylic acids, bile salts, porphyrins, lysozyme, basic peptides, etc. Long-chain fatty acids are toxic for certain bacteria (streptococci, staphylococci, tubercle bacilli) in protein-free media and may play some physiological role in infections of the lung, as they are present in high concentration

[1] Reviewed by Dubos (1954) and Hirsch (1960).

in consolidated lung and in caseous tissue. Certain bacteria are killed more rapidly at low pH in media containing lactic acid than in those containing other inorganic and organic acids, a fact of some interest since lactic acid is a normal metabolic product of phagocytes and a fall in the pH of their cytoplasm is known to take on ingestion of many types of particles (Metchnikoff, 1905).

The only enzyme of leucocytes so far shown to kill bacteria is lysozyme. Its effects are not necessarily limited to those organisms which it can lyse unaided, as chelating agents (Repaske, 1956) or acid (Hirsch, 1960) can extend the range of microbes which are killed by it, with or without apparent lysis.

Two peptides, one rich in lysine and one in arginine, have bactericidal activity *in vitro*, but their importance *in vivo* is doubtful, as they are inhibited by low pH and the presence of nucleic acids. Spermine and spermidine, naturally occurring aliphatic amines, may however play a role in the elimination of infection. In guinea-pig and rabbit the susceptibility of the kidneys to tuberculous infection correlates with the occurrence of the sprmine oxidase required to convert spermine to the derivative active against tubercle bacilli.

These substances are non-specific in the sense that their activity against a particular organism does not depend on a previous immunological experience.

III. C. Role of Antibody

Formation of specific antibodies against components of the microbe or its products is the classical mechanism of

acquired resistance. Owing to the case with which many antibodies can be detected and estimated, a great deal of work has been done on them and their importance in acquired resistance has been axiomatic. Yet the established effects of antibodies on microbial parasites are relatively few.

In the presence of specific antibody and complement, a number of Gram-negative bacteria are lysed and more species are killed without undergoing lysis (Dubos, 1954).

Antibody can promote the ingestion of bacteria by the phagocytes of the host (opsonization).

Antibody may also affect the fate of the bacterium after phagocytosis (Jenkin, 1962).

Bacterial toxins, e.g. of Cl. tetani or C. diphtheriae are inactivated by specific antibody.

Antibody "neutralizes" virus, probably by preventing its entry into cells. [The contrary effect is however reported. Antibody may enhance the uptake of virus particles by cells (Lafferty, 1964) or the plaquing efficiency of a virus in tissue culture (Hawkes, 1964)].

III. D. Cellular Immunity

From the previous section it is clear that antibodies will not curb the activities of a variety of bacteria, notably those which can survive within the phagocytic cells of the host. In tuberculosis, antibodies appear in the serum in most cases, usually in low titre, and no correlation has been established between the level of antibody against any antigen

of the tubercle bacillus and the resistance to infection of the individual or his clinical state (Römer and Joseph, 1910; Raffel, 1955; Boyden, 1958).

In 1905 Metchnikoff suggested that phagocytes of actively immunized animals may be endowed with enhanced phagocytic and digestive activities independent of antibody. This concept of cellular immunity fell into disfavour as the activities of humoral antibodies were more fully explored. However many workers in the field of tuberculosis felt that the mononuclear phagocytes of the reinfected animal hindered the multiplication of the challenge organism in some way, since histological preparations showed that within the cells of immunized animals tubercle bacilli often tended to disappear, whereas in those of normal animals they increased progressively (Rich, 1951).

The first experimental evidence for this concept was Meyer's report (1941) that cells from immune animals destroyed psittacosis virus in the presence of normal serum. In 1942, using an "*in vivo* tissue-culture" system in which cells were injected into the anterior chamber of the rabbit's eye, Lurie showed that tubercle bacilli within immune[1] macrophages did not multiply as rapidly as those in normal macrophages, whereas immune serum exerted an inconstant effect. While this work can be criticised on technical grounds (Mackaness, 1954), the

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[1] In this context the term "immune" is used to refer to cells and serum derived from animals which have previously undergone infection.

findings of Suter (1953) on the bacteriostatic effects of cells in culture are in general agreement with those of Lurie, and later studies have produced evidence of cellular immunity in a number of conditions.

In brucellosis immune macrophages are reported to kill Brucellae more efficiently than normal cells, though in some cases the differences were small and often the cells themselves died after 48 hours (Pomales-Lebrón and Stinebring, 1957; Braun, Pomales-Lebrón and Stinebring, 1958). Holland and Pickett (1958) found that within normal macrophages Brucellae multiplied, increasing approximately 100-fold in 3 days, whereas in immune macrophages their numbers remained constant, but they were not destroyed. Both types of cells survived equally well in tissue culture for 3 days. Macrophages from vaccinated animals showed no bacteriostatic activity and cells derived from animals infected with a rough strain of Brucella restricted the multiplication only of that strain.

The differences between the results of these two groups may be due simply to details of the methods used, levels of parasitization of the cells in culture, etc. The work is technically unsatisfactory to some extent, in that streptomycin was used to control extracellular multiplication of the organisms without evaluation of the possibility that it might penetrate more readily into cells that had been damaged by the ingested parasites or by the manipulations involved in their establishment in vitro. It is significant in this regard that trypsinized cell suspensions from the spleens of animals

previously infected with Brucella underwent rapid cytolysis in culture in the presence of Brucella antigens (Carpenter, Fakuda and Heiskell, 1962). Furthermore the early stages of the bacterium-macrophage interaction were not studied, since the zero values for intracellular populations were determined 2-10 hours after the first contact of cells and organisms.

The complex experiments of Elberg's group, reviewed by Elberg (1960), relate to the degeneration of phagocytic cells in culture on ingestion of Brucellae or tubercle bacilli. Brucella-immune cells degenerate on challenge with Brucellae if the medium contains normal serum, but not in immune serum. The serum factor was not removed by absorption with the same Brucella strain, although no agglutinating or blocking antibodies were detectable after this procedure; the agent involved is thus analogous to a protective factor described in anthrax (Watson et al., 1947). When cells from BCG-vaccinated animals are challenged with virulent tubercle bacilli the findings were similar. Serum from BCG- or Brucella-infected animals protected cells from animals infected with either organism against the degeneration caused by the ingestion of virulent tubercle bacilli. The protective agent was detected in the serum of animals immunized with killed Salm. rutgers (after absorption with the organism) or injected with ovalbumin (alum-precipitated).

These results are difficult to evaluate, since the assay method is not faultless; it is not clear, for example, that total cell counts are always done, and if not cells that

degenerate and lyse will not be taken into account. The authors have assumed that the phenomenon reflects cellular immunity and no attempt has been made to correlate absence of degeneration with intracellular bacteriostasis. This phenomenon of protection may be a manifestation of delayed-type hypersensitivity, since the effects of M. tuberculosis and its culture filtrate on immune cells are indistinguishable.

The results of transfer studies (Fong, Chin and Elberg, 1962) are equally perplexing. Cells from immune animals could confer on normal animals resistance to degeneration. The cells of recipients were protected only in immune serum, but not in their own. Histiocytes which had been frozen and thawed once were still active, but lymphocytes thus treated were not. Protection was detected at 13 days, not at 7 days, and lasted for at least 6 weeks; in one experiment it was transferred serially through 4 recipients at intervals of 13 days. Thus the response in these presumably outbred rabbits resembles the results obtained by Bauer and Stone (see Section II. G.) in inbred guinea-pigs or the "active" sensitization obtained at times by Chase (ibid.).

This work is in such conflict with the findings in other systems that it would be best to withhold assessment until the position is clarified. At present it does not, in my view, constitute evidence either for or against cellular immunity.

Allen (1962) has transferred immunity to tularaemia with peritoneal or splenic cells, the activity of which is

abolished by freezing and thawing. His findings as to the number of cells required and the duration of the transfer in inbred and outbred animals are closely similar to the results of studies in delayed-type hypersensitivity (see Section II. F.). Sever (1960) used similar methods to obtain a slight prolongation of survival under a challenge of virulent tubercle bacilli.

The most clear-cut example of cellular immunity so far described is that active in listeriosis[1]. In mice infected with a sublethal dose of Listeria monocytogenes, the organism multiplies until the fourth day, when the bacterial count in spleen and liver levels off and then begins to fall. During the subsequent two weeks, the peritoneal macrophages of such convalescent animals display a marked ability to kill Listeria. This was shown in a cell-culture system in which extracellular multiplication of the challenge organism was virtually eliminated by thorough washing after parasitization of the cells. Listeria could multiply within 98-99% of normal macrophages, but in less than 1% of immune macrophages.

Antibody appears to play no role either in vivo or in vitro. Immunity cannot be conferred on normal cells in culture by incubation with serum from convalescent animals, nor by urea extracts of immune cells (cf. immunity to Salm.

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[1] This account is based on the reports of Mackaness (1962 and 1964a-d. Listeriosis of mice is described below.

typhimurium, discussed below). Cells frozen and thawed once lose their ability to confer immunity on normal mice. The course of infection in normal mice is not altered by large and repeated doses of serum from convalescent animals.

While the facts just presented make it unlikely that antibody plays a role in the acquired resistance to this disease, it may be premature to draw this conclusion. The example of typhoid is instructive. Salmonella typhimurium is a facultative intracellular parasite which, unlike Listeria monocytogenes, resists phagocytosis. Immunity to typhoid has been considered to be cellular, as cells, and not serum, from convalescent animals were reported to protect normal animals (Ushiba et al., 1959; Saito et al., 1960). However serum collected in the acute phase of the infection and injected in large doses will protect, as will peritoneal cells after freezing and thawing. The active material is apparently a 19S antibody (macro-globulin). Urea extracts of immune cells promote phagocytosis and intracellular destruction of the organism[1].

On the analogy of this work, Jenkin and Rowley (1963) ascribe immunity in diseases such as tuberculosis and brucellosis to a humoral mechanism. Acquisition of resistance, in their view, is due largely to the production of specific antibodies, which affect both the phagocytosis of the parasite

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[1] Immunity to Salm. typhimurium is reviewed by Jenkin and Rowley, 1963.

and its subsequent intracellular career; to this resistance the non-specifically heightened activity of the host's phagocytes (v. infra) will also contribute.

While a cell-associated 19S antibody of the type described in mouse typhoid does not appear to be produced in the immune response to Listeria monocytogenes, it would be unrealistic to suggest that no new types of antibody await appropriate methods of demonstration; the references already made to the work of Boyden (1963a and b) and of Hawkes (1964) and Lafferty (1964) underline this. The participation of an antibody, presumably cell-associated, in the acquired resistance to listeriosis, a resistance which is apparently mediated by the mononuclear phagocytes of the host, cannot be excluded with certainity.

III. E. Specificity of Cellular Immunity

One of the features of the immune response in intracellular infections is lack of specificity. Tuberculous or BCG-vaccinated animals have been found partially resistant to Brucella abortus (Pullinger, 1936; Sulitzeanu et al., 1962), to Pasteurella pestis (Girard and Grumbach, 1958). Brucella-infected animals show increased resistance to tuberculosis (Nyka, 1956) and in both experimental and clinical situations leprosy protects against plague (Girard, 1951). Such cross-resistance may be termed "non-specific", or it may be attributed to the more rapid mobilization of phagocytes (Pullinger, 1936) or to the increased activity of the reticulo-endothelial system known to

occur in tuberculosis (Lurie, 1939).

Mackaness (1964) finds that in the first two or three weeks after Listeria infection mice are more highly resistant not only to a challenge of Listeria, but also to Brucella abortus. At a later period, when resistant macrophages have disappeared from the tissues, a challenge inoculum of Listeria grows for a period in the spleen and liver and then is rapidly killed by what is apparently an anamnestic cellular response. Brucella however multiplies normally in such animals, without exciting an accelerated immune response.

In normal mice Brucella grows at a constant rate for 10-12 days. During this period the growth of Listeria in the tissues or its survival in peritoneal cells is unaffected. In the subsequent 12-14 days, the numbers of Brucellae in the spleen first level off and then fall somewhat. In this phase of the infection, an inoculum of L. monocytogenes is rapidly destroyed in the spleen and peritoneal macrophages show prompt and marked bactericidal activity for this organism. In phase three of the Brucella infection, ca 4 weeks after inoculation, the population of Brucella in the spleen remains at a constant level, and Listeria can multiply in this organ, but at a reduced rate, without however eliciting an anamnestic response.

In related experiments the response of BCG-vaccinated mice to Listeria infection was investigated. Mice were infected with BCG. Fourteen weeks later, half of these mice again received a dose of BCG, the same dose being given to half the control group of uninfected mice. Then all were

(To face Page 56).

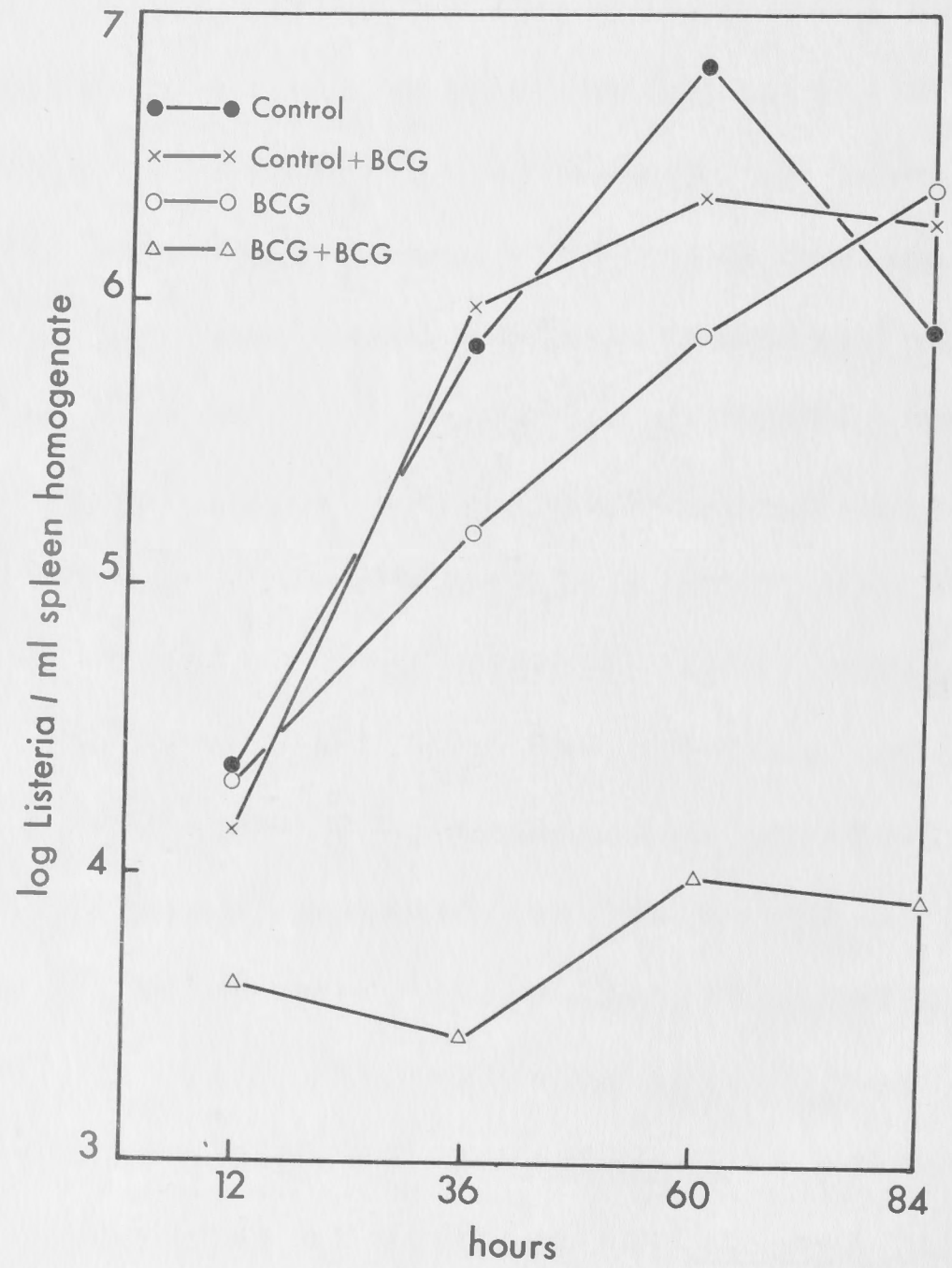


Figure 1.

Growth curves of L. monocytogenes in the spleens of normal or BCG-sensitised mice given an intravenous injection of BCG 3 days prior to challenge with L. monocytogenes (●----●) normal mice; (o----o) BCG-sensitised mice; (x----x) normal mice given BCG prior to challenge; (Δ ---- Δ) BCG-sensitised mice given BCG prior to challenge. Means of 6 mice per group.

infected with Listeria. The fate of this organism in the spleen is shown in Fig. 1. It is apparent that Listeria multiplied freely in all animals except those which, being previously sensitized to BCG, had received a second dose of this organism. Thus BCG, but not Listeria, could elicit an anamnestic immune response to which Listeria was vulnerable.

Hence it seems clear that the macrophages of mice can acquire an enhanced capacity to kill intracellular parasites, and that, once acquired, this capacity lacks specificity. Induced by one organism, it is active against others, but once it has partly decayed, so that immune macrophages are not immediately available but can only be produced by an anamnestic response, this response can only be evoked by the organism against which resistance originally arose. Thus the response is specific to a degree and so presumably is the result of an immunological reaction.

Mackaness suggests that during the original infection the macrophages of the host become sensitized, perhaps in the fashion of delayed-type hypersensitivity, and that on further contact with the antigen these sensitized cells are altered in some way. The phagocytes of tuberculous animals are known to be stimulated both in the uptake of foreign particles (Lurie, 1939) and in general metabolic activity (Allison, Zappasodi and Lurie, 1962) and they also possess an increased content of certain enzymes (Grogg and Pearse, 1952).

The numbers of organisms in the tissues could thus exert an influence on the level of immunity. When the

population falls below a certain size (cf. phase 3 of the Brucella infection described above) the stimulus is insufficient. Such a mechanism could account for the finding in tuberculosis (Levy et al., 1961) that a challenge organism proliferates initially in the immunized animal to the same extent as in the normal.

Jenkin and Rowley (1963) would deny these cellular reactions immunological status because of their lack of specificity. The cogency of this objection is not entirely clear, for it could equally well be argued that other classical manifestations of immunological reactions are non-specific, if only their end-effects are considered. The combination of antigen with antibody has defined the concept of immunological specificity; the observation that in animal tissues many specific antigen-antibody reactions lead to the release of the same substance, histamine, affords us an analogy with the reactions of cellular immunity already described. In the latter case we do not yet know what is the process set in train by the interaction of the micro-organism and the specifically sensitized cell (an obvious possibility is the production of some antimicrobial factor, such as was described in Section III. B.). Nevertheless, the finding that the initiation of an anamnestic response in cellular immunity requires the participation of

the organism against which the cells originally acquired bactericidal powers suffices to categorize this phase of the process as immunological.

## IV. LISTERIOSIS

*Listeria monocytogenes* was first described in 1926 by Murray, Webb and Swan, who isolated it from rabbits in a colony overcome by an epidemic disease. It has subsequently been found in numerous species of animals and is now recognised as a cause of disease in man[1]. The natural history of the infection is not well understood. The reservoir is unknown, so that the source of the sporadic outbreaks of listeriosis in animals (the disease is of some economic importance, since it attacks sheep and cattle) and of the infrequent cases in man has not been determined. The organism can survive in moist soil for six months and has been isolated from silage.

*Listeria monocytogenes* is a small, Gram-positive rod related to the *Corynebacteria* and to *Erysipelothrix*. There are five serological types distinguished by their O and H antigens. Serological type does not appear to correlate with either geographical or host range.

The pattern of disease is polymorphic and varies with the host. In rodents there is a generalized infection with massive involvement of the liver; in ruminants the central nervous system is chiefly affected and encephalitic and meningitic syndromes result, e.g. "circling disease" of sheep. In pregnant animals it produces stillbirth or abortion.

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[1] Listeriosis has been reviewed by Hoeprich (1958) and Seeliger (1958), on whose writings the description of listeriosis in animals other than the mouse is largely based.

The organism derives its name from the marked monocytosis of the peripheral blood which infection produces in rabbits, as was noted by Murray et al. (op. cit.). While this phenomenon is not a necessary accompaniment of listeriosis in other hosts, the monocyte-macrophage cell complex is intimately associated with the manifestations of this disease.

The characteristic lesion of listeriosis is a small focal necrosis, which is seen as a small white spot on the surface of such organs as the liver and spleen. Histologically a focus in the liver consists of a small round zone of open-meshed reticulum containing large mononuclear cells and nuclear debris. The focus is bordered by accumulations of Listeria monocytogenes. Similar lesions are found in spleen, lung and kidney.

The genesis of these foci was studied in the tissues of mice by Mackaness (1962). Twenty-four hours after intravenous infection with $5.6 \times 10^4$ listeriae discrete foci were readily found in the liver and, with greater difficulty, in the spleen. In the liver each microscopic accumulation consisted of neutrophils and mononuclear cells, the former predominating. Organisms, if present, appeared to be within cells. At 48 hours the lesions were larger; a central core of neutrophils was surrounded by a zone of mononuclear phagocytes and it was in this region that the organisms were seen. Over the following two days the focus enlarged, the central neutrophils became pyknotic and macrophages accumulated round the periphery and invaded the inner region. Most of the

mononuclear cells appeared healthy and resembled epithelioid cells somewhat. The numerous listeriae seemed to be in process of alteration, since many were now cocco-bacillary in form and were in most cases Gram-negative. On the fifth and sixth days a marked drop in the numbers of stainable organisms had taken place. The focus still consisted of an aggregation of healthy mononuclear phagocytes.

The host-parasite relationship in listeriosis in mice was examined both *in vivo* and *in vitro* (Mackaness, 1962). Reference has already been made(p. 52 ) to the capacity of macrophages from previously infected animals to inactivate *L. monocytogenes* when the phagocytes were maintained in culture and were unaided by serum factors. It is now proposed to discuss briefly some features of the disease process *in vivo*.

The wild strain of *L. monocytogenes* used (N.C.T.C. 7973) is of low virulence, the $LD_{50}$ for mice being $7.2 \times 10^7$ by the intraperitoneal route. If this organism is used to parasitize monolayers of peritoneal macrophages from normal mice, only ca 0.02% of the bacteria are found capable of intracellular survival and multiplication to the point where the phagocyte is killed and a small plaque appears in the monolayer. Organisms re-isolated from such macrophage cultures show a greatly enhanced ability to survive within peritoneal macrophages *in vitro*; 70-95% of these organisms can now resist the antibacterial activities of normal macrophages. The virulence of the organism is also enhanced,

the $LD_{50}$ for intraperitoneal challenge now being $5.3 \times 10^5$. Conversely, one passage in mice increases the virulence by the same factor (approximately 200-fold) and the great majority of these mouse-passaged organisms can survive intracellularly. (Only virulent organisms are used in the work to be described below).

If ca $10^5$ viable units of L. monocytogenes are injected into healthy mice by the intravenous route, the bulk of the inoculum can be recovered from the liver and spleen soon afterwards. When the bacterial population of these organs is enumerated at intervals, Listeria is found to grow in liver or spleen in log.-linear fashion, the generation time being approximately 5 hours. (If this calculation yields the actual intracellular generation time, then the process of division is considerably slower in vivo than in vitro; in static culture the generation time is about one hour at 37°C.). After three days bacterial growth ceases and a rapid inactivation ensues; the numbers of listeriae found in spleen and liver decline sharply in the following 6 days.

When animals which have survived a primary infection with $10^4$ to $10^5$ listeriae are challenged, they are found to be highly resistant, surviving infection with 1000 to 1000 $LD_{50}$s. The bacterial counts in the spleens and livers of such mice fall precipitately, so that after two days these organs contain only 1/10,000 as many listeriae as are present in normal tissues. It is clear therefore that convalescent animals possess a potent antibacterial defence. The nature of this acquired

resistance has been discussed in some detail above ( p.52 ).

The occurrence of delayed-type hypersensitivity in listeriosis of rabbits and guinea-pigs was reported by Markham (1954). Skin reactions were elicited with a sonically disrupted bacterial suspension. This observation was confirmed (Mackaness and Ackerman, 1962). Mice infected with L. monocytogenes show delayed footpad reactions to injections of culture filtrate as early as 4 days after inoculation with the organism. The emergence of hypersensitivity therefore coincides with the development of acquired resistance, since at this time the bacterial population of the organs ceases to multiply rapidly and soon starts to decrease.

There have been few previous investigations of the nature of the acquired resistance to Listeria. Julianelle (1941) could find no evidence that antibodies played any role, a conclusion with which Osebold and Sawyer (1957) were in agreement. There is a good deal of evidence that most killed vaccines confer no protection (e.g. Graham, Morrill and Levine, 1940), although Eveleth (1963) reports his "clinical impression" that suspensions of Listeria killed by ultra-violet irradiation are more effective. Osebold and Sawyer (op. cit.) and Hasenklever and Karakawa ( 1957 ) showed that active immunity was conferred on mice by a previous infection with Listeria.

The capabilities of macrophages from convalescent sheep were investigated by Njoku-Obi and Osebold (1962).

They found that macrophages from previously infected animals could restrain the multiplication of Listeria to some extent when maintained in serum from immune animals but not when the medium contained normal serum only. Extracellular multiplication of the organisms was not controlled (in the studies of Mackaness the extracellular bacterial population was reduced to a minimum by extensive washing with a jet of saline), and the levels of parasitization in the cell cultures were extremely high. Most of the cells in their experiments were destroyed within 20 hours; even in cultures of immune macrophages in immune serum only 40% of the cells survived for this period. The findings are difficult to interpret and may reflect both intracellular bacteriostasis and delayed-type hypersensitivity (cf. the discussion of the work of Elberg's group on brucellosis and tuberculosis, pp.50-51).

# CHAPTER I.

## MATERIALS AND METHODS

### EXPERIMENTAL ANIMALS

Mice only were used. Apart from one experiment in which two inbred strains, Bagg and CBA, appeared, all work was carried out in an outbred strain originally obtained from the Walter and Eliza Hall Institute, Melbourne, and referred to as Hall mice where necessary. Mice were bred in the Animal Breeding Establishment of The John Curtin School of Medical Research. They were fed a cubed basic diet without antibiotic additives and given water ad libitum. The cages used contained up to 25 animals.

In various experiments the age of the mice ranged between 7 and 12 weeks. In any one experiment only mice of the same sex appeared and their ages differed by a maximum of 2 weeks. In order to avoid any effects due to variation in the cages of stock mice (each of which represented the males or females from 6-10 litters only), the required number of stock cages was selected at the onset of the experiment. Mice from each stock cage were then distributed equally among the experimental cages.

Hall mice are of seven readily distinguishable colours. By means of two dyes, carbolfuchsin (1% in 10% ethanol in water) and picric acid (a saturated solution in 40% ethanol in water) as many as 20 individuals could be

identified in the one cage.

## BACTERIOLOGICAL METHODS

The bacterial culture media in routine use were brain-heart infusion (BHI) broth and brain-heart infusion agar (Difco Labs, Michigan). All cultures were incubated at 37°C.

Isolation of L. monocytogenes from Organs. Mice were killed by cervical dislocation and the abdominal skin was reflected so as not to contaminate the upper abdominal wall. The peritoneal cavity was entered and the organ to be examined was dissected free. It was then placed in a homogenizer tube containing 5.0 ml. of BHI medium (when spleen and liver were homogenized together, the tube contained 10 ml. of medium). The tissue was then dispersed for 1-2 minutes with a motorized teflon pestle. If it was desired simply to isolate L. monocytogenes from the spleen, one drop of the resulting suspension was added to 10 ml. of BHI medium and the culture incubated overnight.

Bacterial Enumeration. A suspension of the required organ was prepared as just described. Serial 10-fold dilutions were then made in BHI medium and 0.1 ml. of each was plated on well-dried BHI agar in quadrant plates. Colony counts were made after incubation for 24 hours at 37°C. The lowest practicable dilution was chosen for counting, i.e. that dilution yielding 10-100 colonies.

## Preparation of the Stock Virulent Strain of *L. monocytogenes*.

A primary culture of a virulent strain was prepared from a spleen homogenate; the donor of the spleen had been infected 3 days previously with a virulent organism, i.e. one that had already undergone mouse-passage (pp. 61-2). The culture was grown overnight, the organisms were freeze-dried in ampoules and kept at -20°C.

Preparation of Virulent Inocula. An aliquot of the above stock culture was grown for 5-6 hours and the resulting suspension was injected intravenously into mice in a dose of ca $10^5$ organisms. After 48-72 hours the organism was isolated from the spleen as described above and re-injected into mice. After this second passage it was considered ready for use.

The organism was maintained by repeated passages in normal mice at intervals of 3-5 days. After a total of ca 8 passages the strain in use was discarded and a fresh virulent culture prepared from the freeze-dried stock. It was hoped by these means to ensure that the characteristics of the strains of *Listeria* used would remain fairly stable throughout the work. Cultures for experimental purposes were always primary isolates from the spleen; the suspension was used within 24 hours of removal of the spleen and was not subcultured.

When it was desired to infect a group of mice, a suspension of a virulent strain was dispersed by 3-5 seconds' treatment with an M.S.E. magnetostrictive ultrasonicator and the number of organisms determined in a Petroff-Hauser

bacterial counting chamber. The appropriate dilution was then made in Hanks balanced salt solution containing 0.1% of bovine serum albumin. The number of organisms in the suspension was checked by a viable count. In most cases the correlation between the chamber count and the viable count was good, the latter being in general 50-200% of the chamber count and often lying within closer limits still.

Technique of Inoculation. Before intravenous injection mice were placed in an incubator at 40°C. for 5-10 minutes. The veins of the tail could then be easily entered. Intraperitoneal injections were made into the lower left quadrant of the abdomen and subcutaneous injections into the flanks. For most purposes a volume of 0.1 ml. was injected. (In a challenge, 0.2 ml. was given). Bacterial suspensions were injected from 1 ml. tuberculin syringes.

Where groups of mice are compared after infection, it is implied, unless otherwise stated, that all received the appropriate dose of Listeria from one bulk suspension of organisms diluted as necessary.

$LD_{50}$ Determination. Groups of 10 mice were used. Five-fold dilutions of the Listeria suspension were injected in 0.2 ml. Deaths were recorded for 10 days.

Preparation of Listerin. The term "listerin" has been coined, on the analogy of tuberculin, to describe the culture filtrate used to elicit footpad reactions. To prepare this material, a concentrated solution of BHI medium with added Bacto-Tryptose (Difco) was dialysed against distilled water.

Each litre of the final medium contained the dialysable material from 40 G of BHI medium and 10 G of Bacto-Tryptose. Glucose (40 G/1.) and ammonium chloride (10 G/1.) were added after dialysis. Eight litres of the dialysate medium were Seitz-filtered into a large vessel and inoculated with 200 ml. of an overnight culture of a virulent strain of L. monocytogenes likewise in dialysate medium. The contents of the vessel were agitated and oxygenated by a stream of sterile air and the pH was kept within the range 7 to 8.5. At intervals aliquots were withdrawn and the pH determined with a Beckman pH meter (Model H2). The reaction of the culture was then adjusted with sterile sodium bicarbonate. After 20 hours the bacterial count had reached $10^{10}$/ml. The culture vessel was then transferred to the cold room and the bacteria spun down as rapidly as possible. The organisms were freeze-dried. The supernate was sterilized by passage through a "Millipore" filter (Millipore Filter Corp., Bedford, Mass.) of 0.45μ mean pore diameter and kept at -70°C. This material constituted the listerin used throughout the work on delayed-type hypersensitivity. Two different batches of listerin were prepared in this way; the results of an assay of the biological activity of each preparation are reported in Chapter II. A concentrate of listerin was prepared by freeze-drying the material and redissolving it in distilled water to one fifth of the initial volume.

"Listeria antigens". Five grams of freeze-dried organisms were suspended in 50 ml. of 0.01 M phosphate buffer,

pH 7.4, and disrupted by treatment in a sonic oscillator (Raytheon Manufacturing Co., Waltham, Mass.) at maximum output for 45 minutes at 4°C. The material was then diluted to 200 ml. with the same buffer, centrifuged for 1 hour at 5000 G and the supernatant was diluted with 4 volumes of listerin and passed through an 0.45μ "Millipore" filter. The final product (referred to below as "a preparation of Listeria antigens") was kept at -70°C until required.

Microsomal and Cytoplasmic Fractions of L. monocytogenes

Freeze-dried organisms were disrupted as just described. After dilution to 200 ml. the material was centrifuged once at 2500 G for one hour and three times at 13, 500 G for 10 minutes, the deposit being discarded on each occasion. The final supernate was centrifuged at 143, 000 G for three hours. The deposit from this centrifugation constituted the microsomal fraction and the supernate the cytoplasmic fraction.

Preparation of Vaccines. The starting material in each case was the freeze-dried preparation of L. monocytogenes. Bacteria were mixed with each of the following sterilizing agents in the ratio of 2 G dry weight to 40 ml.; acetone, 70% ethanol in water, a mixture of diethyl ether and water 1:8, 0.2% formalin in water, 5% phenol in water, 10% trichloracetic acid in water. The resulting suspensions were agitated at 4°C for 24 hours and kept at this temperature for a further 6 days.

The preparations were freed of acetone and ethanol by repeated centrifugation and re-suspension in buffered saline. The remaining agents were removed by dialysis against distilled water at 4°C in dialysis sacs sterilized by immersion in 70% alcohol.

For heat sterilization, freeze-dried organisms were suspended in water, enclosed in sealed tubes and heated by total immersion in a water bath at 70°C for 40 minutes and 100°C for 5 minutes.

The number of bacteria per gram in the freeze-dried preparation was determined by suspending the organisms in water and counting the suspension in a Petroff-Hauser chamber. Before use each vaccine was diluted so as to contain $10^{10}$ organisms/ml. or the equivalent in bacterial substance (ether and phenol produced lysis of the suspensions). The sterility of the vaccines was checked by culture on solid and liquid medium for 3 days at 37°C.

Bacillus Calmette-Guerin (BCG). Suspensions containing $10^{10}$ organisms per ml. were prepared from the freeze-dried vaccine supplied by Commonwealth Serum Laboratories, Melbourne.

Miscellaneous Reagents

Silicic Acid. Silicic acid (325 mesh, Bio-Rad Laboratories, Richmond, Cal.) was suspended in water and the coarser material removed by sedimentation. Microscopic examination of the resulting suspension showed that few of the particles were as much as 5μ in their longest dimension. The

preparation was autoclaved and the pH was adjusted to 7.0 with 10 N sodium hydroxide.

Adjuvants. Freund's incomplete adjuvant (mineral oil + suspending agent), and Freund's complete adjuvant (mineral oil + suspending agent + killed M. butyricum) were obtained from Difco Laboratories.

Hanks Balanced Salt Solution was prepared as indicated by Bazeley and Thayer (1954) and supplied by the Department of Microbiology, J.C.S.M.R. It contains:

| | | | |
|---|---|---|---|
| NaCl | 8.0 G/l | $CaCl_2$ | 0.14 G/l |
| KCl | 0.4 G/l | $Na_2HPO_4$ | 0.06 G/l |
| $MgSO_4.7H_2O$ | 0.1 G/l | $KH_2PO_4$ | 0.06 G/l |
| $MgCl_2.6H_2O$ | 0.1 G/l | Glucose | 1.0 G/l |

Three ml. of 1.4% $NaHCO_3$ are added to each 100 ml. before use.

Buffered Saline. Isotonic phosphate buffer, pH 7.4, was prepared as described by Magnusson et al. (1958). It contains potassium dihydrogen phosphate (1.45 G/l), disodium hydrogen phosphate dihydrate (7.6 G/l) and sodium chloride (4.8 G/l).

Pyrogen-free Saline. Sodium chloride injection B.P. was obtained from Commonwealth Serum Laboratories.

Oxytetracycline. Terramycin (Animal Formula) Soluble Powder (Pfizer Corp.) was dissolved in water in a concentration of 36 G/l. This yields a solution containing 2 G/l of pure oxytetracycline. The antibiotic was administered

to mice in their drinking water.

Presentation of Experimental Results. The methods of reporting tests for delayed-type hypersensitivity will be discussed in Chapter II. The level of acquired resistance is usually assessed by determining the numbers of bacteria in the spleen 1-3 days after the intravenous injection of a challenge inoculum. In a number of experiments the "immunity index" was used. Challenge and spleen count were carried out as usual for both test and control (uninfected) animals. From these data the "immunity index" was calculated for each mouse in the test group.

$$\text{Immunity Index} = \text{Log.} \frac{(\text{Mean spleen count of normal controls})}{(\text{Spleen count of individual mouse})}$$

e.g. Mean spleen count of controls = $6 \times 10^6$ organisms/ml.

Spleen count of mouse A = $3 \times 10^3$ " "

Immunity index of mouse A = log. $(6 \times 10^6 / 3 \times 10^3)$

= 3.30

The "immunity index" is very similar to the "protection index" used by Sulitzeanu (1955).

Statistical Methods. The formulae used in carrying out statistical tests are given.

$$\text{Mean} = \frac{\Sigma x_i}{n}$$

$$\text{Variance} = \frac{\Sigma x_i^2}{n} - \bar{x}^2$$

(This formula gives a biassed value for the variance, but this is allowed for in the formulae used for *t* and *F* tests).

Student's *t* test:

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{n_x s_x^2 + n_y s_y^2}} \cdot \sqrt{\frac{n_x n_y (n_x + n_y - 2)}{(n_x + n_y)}}$$

*F* test:

$$F = \frac{n_x s_x^2/(n_x - 1)}{n_y s_y^2/(n_y - 1)}$$

Chi-square test:

$$\text{Chi-square} = \sum \frac{(o_i - e_i)^2}{e_i}$$

($o_i$ = any observed frequency; $e_i$ = the coresponding expected frequency)

Transformation. The results of bacterial counts on organs are transformed by taking the logarithm of each value. This brings the distribution of the values closer to the normal distribution. Tests for significance are applied to the transformed data. (Means given in the text are calculated from the untransformed values and are arithmetic).

In order to decide whether two values for mean

spleen count or mean footpad reaction differed significantly, the *t* test was used. Occasionally, as when it was desired to combine two groups for further statistical analysis, an *F* test was applied as well.

Error of Footpad Measurements. The method of Nissen-Meyer, Hougen and Edwards (1951) was used. The differences between the readings for right and left feet were found (see Table 4 of Chapter II) and the variances of these differences ($s_d^2$) calculated. The experimental error is then $\sqrt{\frac{1}{2} s_d^2}$.

The value of the $LD_{50}$ was calculated by the Spearman-Kärber method as given by Finney (1951). The significance of differences between $LD_{50}$s was found by the method used by Osebold and Sawyer (1957). The 1% confidence limits were taken to lie three standard deviations on either side of the mean.

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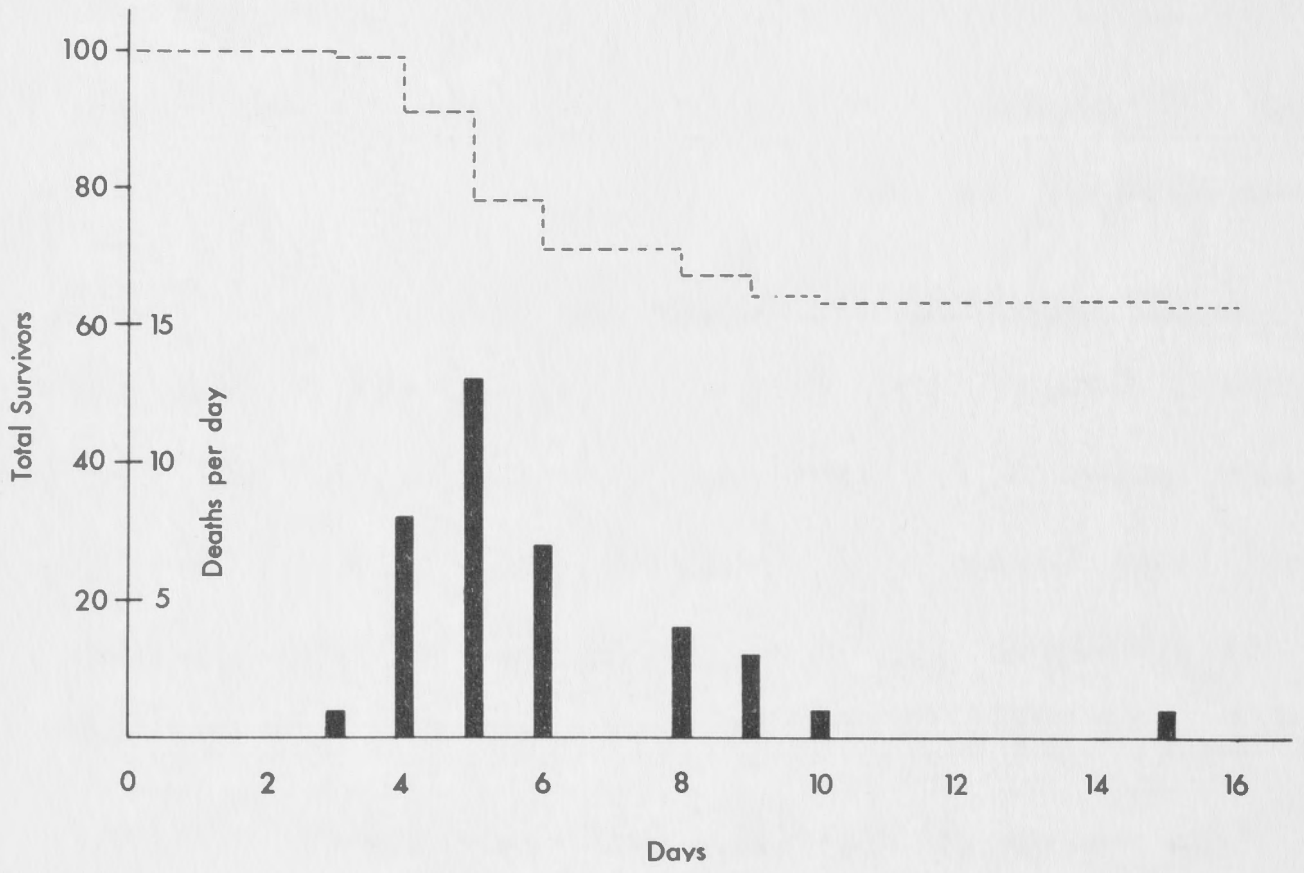


Figure 2.

Survival curve and daily death-rate of mice after intravenous infection with $10^5$ listeriae on Day 0.

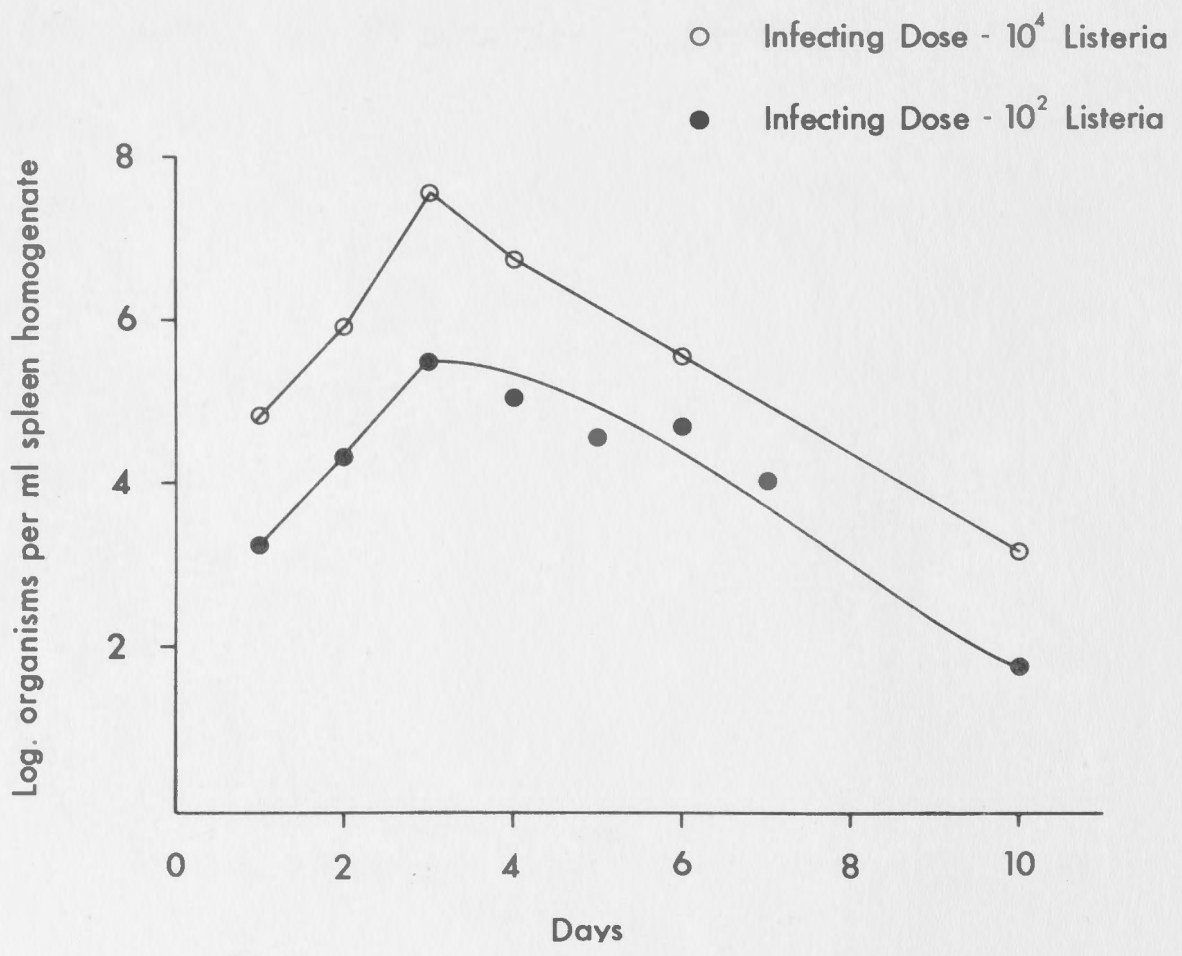


Figure 3.

Results of daily spleen counts on mice after intravenous infection with $10^4$ and $10^2$ listeriae.

## CHAPTER II.

## EXPERIMENTAL INVESTIGATION OF LISTERIOSIS IN MICE

The experimental infection of mice with *Listeria monocytogenes* has been discussed in Section IV of the Introduction. Some further details will be presented here, followed by an account of work which has contributed to the development and assessment of the two principal methods used in these investigations.

Clinical Course. Inoculation of normal mice with *L. monocytogenes* by the intravenous or intraperitoneal routes leads to a disseminated infection with fever and considerable weight loss but few distinctive features. A purulent conjunctivitis is however an almost invariable manifestation in animals undergoing infection initiated by doses of 0.5 $LD_{50}$ or more.

If normal mice receive ca $10^5$ listeriae intravenously, most are little affected until the third day, when they will appear sick and weight loss begins to be evident. The mortality curve of a group of mice infected intravenously with $10^5$ viable units of *L. monocytogenes* is given in Figure 2, where it will be seen that most deaths occur between days 4 and 9. Of the survivors 6 appeared wasted and lethargic at day 15, and 3 still showed conjunctivitis. The rest were apparently fully recovered. In this experiment only one mouse showed evidence of neurological involvement, being paralysed in both hind legs from the tenth day on. Partial or complete

paralysis of one or more limbs, more often the hind limbs, is not uncommon, and occasionally 10-15% of the mice in an experiment will be thus affected, either temporarily or permanently.

No convalescent animal which appears at all sick is used for further work, as such animals rarely manifest any degree of delayed-type hypersensitivity and L. monocytogenes can often be isolated from their spleens two or three weeks after the initial infection.

Growth Curve of a Small Inoculum. When mice are infected intravenously with a small dose, e.g. $10^2$ organisms, the form of the growth curve in the spleen resembles that already described for inocula of $10^4$ or $10^5$ organisms. In Figure 3 are given the results of daily spleen counts on normal animals which had received, respectively, $1.1 \times 10^2$ and $9.3 \times 10^3$ listeriae by the intravenous route.

It is of interest that the evolution of an infection initiated with a lower dose shows apparently no greater tendency to chronicity than does a more severe infection. While the point has not been specifically examined, none of the later work (for example, on the duration of acquired resistance) offers any suggestion that Listeria monocytogenes can persist in an apparently healthy spleen. Foci of infection have not been sought in other organs of animals which had seemingly recovered completely from their initial infection.

The 50% Lethal Dose. Determinations of the $LD_{50}$

by the intravenous and intraperitoneal routes were carried out as described in Chapter I and the results appear in Table 1.

TABLE 1

The $LD_{50}$ for Listeria Infections initiated by the Intravenous and Intraperitoneal Routes

| Age and sex of mice | Route of Challenge | $LD_{50}$ |
|---|---|---|
| Male, 8 weeks | Intravenous | $3.2 \times 10^5$ |
| Female,10 weeks | " | $2.4 \times 10^5$ |
| Male, 10 weeks | Intraperitoneal | $3.6 \times 10^6$ |

The $LD_{50}$ by the subcutaneous route was not determined, but it is well in excess of $10^7$, probably not far short of $10^8$ organisms. In one experiment a dose of $4.4 \times 10^6$ subcutaneously produced no symptoms in a group of normal mice.

Assessment of Acquired Resistance. As a measure of the level of acquired resistance I have taken in most cases the numbers of organisms surviving in the spleen one to three days after challenge, according to the conditions of the experiment. These "spleen counts" are readily carried out and the statistical methods available for their evaluation (see Chapter I) are more discriminating than those which can be used in survival studies. An attempt was made to compare the two

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TABLE 2

Comparison of Level of Acquired Resistance as Measured by Spleen Count and by $LD_{50}$

| Group | Inoculum and route of inoculation | Mean Spleen Count (organisms/ml. homogenate) | | $LD_{50}$ |
|---|---|---|---|---|
| | | Number of mice | Mean | |
| I | Nil | 5 | $9.7 \times 10^6$ | $2.4 \times 10^5$ |
| II | $10^4$: Intra-venous | 9 | $4.3 \times 10^3$ | $1.1 \times 10^7$ |
| III | $10^4$: Intra-peritoneal | 10 | $3.6 \times 10^5$* | $4.7 \times 10^6$ |
| IV | $10^6$: Intra-peritoneal | 8 | $10^5$ | $2.3 \times 10^7$ |

* The variance of Group III was unusually small, thus the mean spleen counts of groups III and IV differ significantly (see Table 3 ). In other experiments the difference between the two groups was greater.

TABLE 3

Statistical Significance of Differences in Level of Acquired Resistance shown in Table 2

| Groups Compared | Significance of Difference between: | |
|---|---|---|
| | Mean Spleen Counts | $LD_{50}$s |
| II and III | $<0.001$ | n.s. |
| II and IV | $<0.01$ | n.s. |
| III and IV | $<0.005$ | $<0.002$ |

methods and decide to what extent the results provided by one paralleled the other.

Mice ~~F, 8 weeks~~ received the following doses of L. monocytogenes by the route indicated:

$1.5 \times 10^4$ intravenously

$1.5 \times 10^4$ intraperitoneally

$1.5 \times 10^6$ "

Nil

Mice from each group were challenged by the intravenous route with $7.8 \times 10^4$ listeriae. Spleens were removed 48 hours later and bacterial counts performed, the results appearing in Table 2. At the same time the 50% lethal dose was determined for each of the above groups, using fivefold decrements, the largest dose given being $5 \times 10^8$ organisms. The $LD_{50}$s are also reported in Table 2 (the calculations are discussed in Chapter I).

The levels of acquired resistance in each of the immunized groups were compared, using each method in turn, and the statistical significance of the differences observed is indicated in Table 3; the resistance manifested by each of the previously infected groups is obviously far higher, measured by either method, than that of the normal animals.

The results of the foregoing experiment are entirely consistent with other work, not reported here, in which the viable spleen count or survival data have been used to measure the level of resistance. The results show that animals which differ markedly in the bactericidal powers of their spleens may

manifest equal degrees of resistance when this is assessed for the animal as a whole in less specific fashion. The discrepancies revealed in the comparison are of considerable interest. The explanation is probably to be found in the differential stimulation of certain tissues on infection by a particular route. The level of acquired resistance of splenic tissue is much higher after intravenous than after intraperitoneal infection by the same number of organisms, but the resistance of the whole animal is comparable in either case. (A similar disparity between the results of spleen count and $LD_{50}$ determination will be discussed in the section on desensitization in Chapter VII). Hence it appears that the index of acquired resistance obtained from bacterial counts in a given organ does not necessarily reflect with complete fidelity the capacity of the whole animal to survive a challenge infection. However, where the same route was used to initiate the primary infection in all the groups of animals under examination, as in Groups III and IV of Table 2, there is no discrepancy, nor would one be expected on theoretical grounds, unless the inoculum were so large as to alter the character of the pathological processes involved.

Assessment of Delayed-Type Hypersensitivity in Mice

The realization that delayed hypersensitivity reactions can be elicited in mice is comparatively recent. Earlier workers, e.g. Gerstl and Thomas (1941) failed to deomonstrate skin reactivity to PPD in mice infected with tubercle bacilli.

Fenner (1948) showed that the injection of virus into the footpads of mice infected with ectromelia produced delayed reactions maximal at 24-48 hours and declining thereafter. And subsequently others succeeded in revealing systemic reactions (Hart, Long and Rees, 1952; Kirchheimer and Malkiel, 1953) and then local reactions (Gray and Jennings, 1955) to tuberculin and PPD, Gray and Jennings showed that, in mice inoculated with virulent tubercle bacilli or BCG, injection of Old Tuberculin or PPD into the dermis of the footpad produced an inflamed swelling, marked at 24 hours and 48 hours. They recorded their observations as positive, intermediate or negative, on the basis of gross appearance. In subsequent work (Gray, Graham-Smith and Noble, 1960) measurements were made with ≠ dial-gauge calipers and "positive" was defined as any reaction 0.2 mm. greater than the average of the controls.

Selbie and O'Grady (1954) injected tuberculin into the thighs of tuberculous mice and measured the increase in mediolateral diameter of the thigh, thus establishing the presence of delayed hypersensitivity. Crowle (1959a) determined the density of the lungs after an intravenous challenge dose of tubercle bacilli and found an increase in tuberculous mice. This method is of limited application, even if it should prove to be a true measure of delayed-type hypersensitivity (see Section II.B. of the Introduction).

Crowle (1959b) has reviewed the methods of assessing the tuberculin reaction in mice. Of the six tests evaluated,

the footpad reaction, the intra-cutaneous and intra-muscular tests, lung density, systemic reactions and change in body temperature, the first three were found to be superior in reliability, sensitivity and ease of execution.

In order to make any but the crudest comparisons between the hypersensitivity levels of groups receiving different treatments, quantitative methods are essential. Of the methods mentioned above the footpad reaction was chosen for further study, as it seemed likely to be superior to others in speed and simplicity of execution and in ease of quantitation.

## Technique Used to Determine Hypersensitivity Levels in Mice

The preliminary experiments confirming the occurrence of delayed hypersensitivity in listeriosis (Mackaness and Ackerman, 1962) were carried out with a simple technique. The right footpad of the mouse was injected with 50 µl. of culture filtrate using a tuberculin syringe and a 28G needle. Measurements of the dorso-ventral footpad thickness were made at intervals with dial-gauge calipers. The bulk of the initial injection was such as to preclude any measurement of the immediate hypersensitivity reaction (e.g. 3 hours after injection).

This technique was modified as follows. The mouse is held in a wire cylinder and the right foot (sole uppermost) is presented to a micrometer syringe (Agla, Burroughs Wellcome) held horizontally in a clamp and armed with a 30G needle

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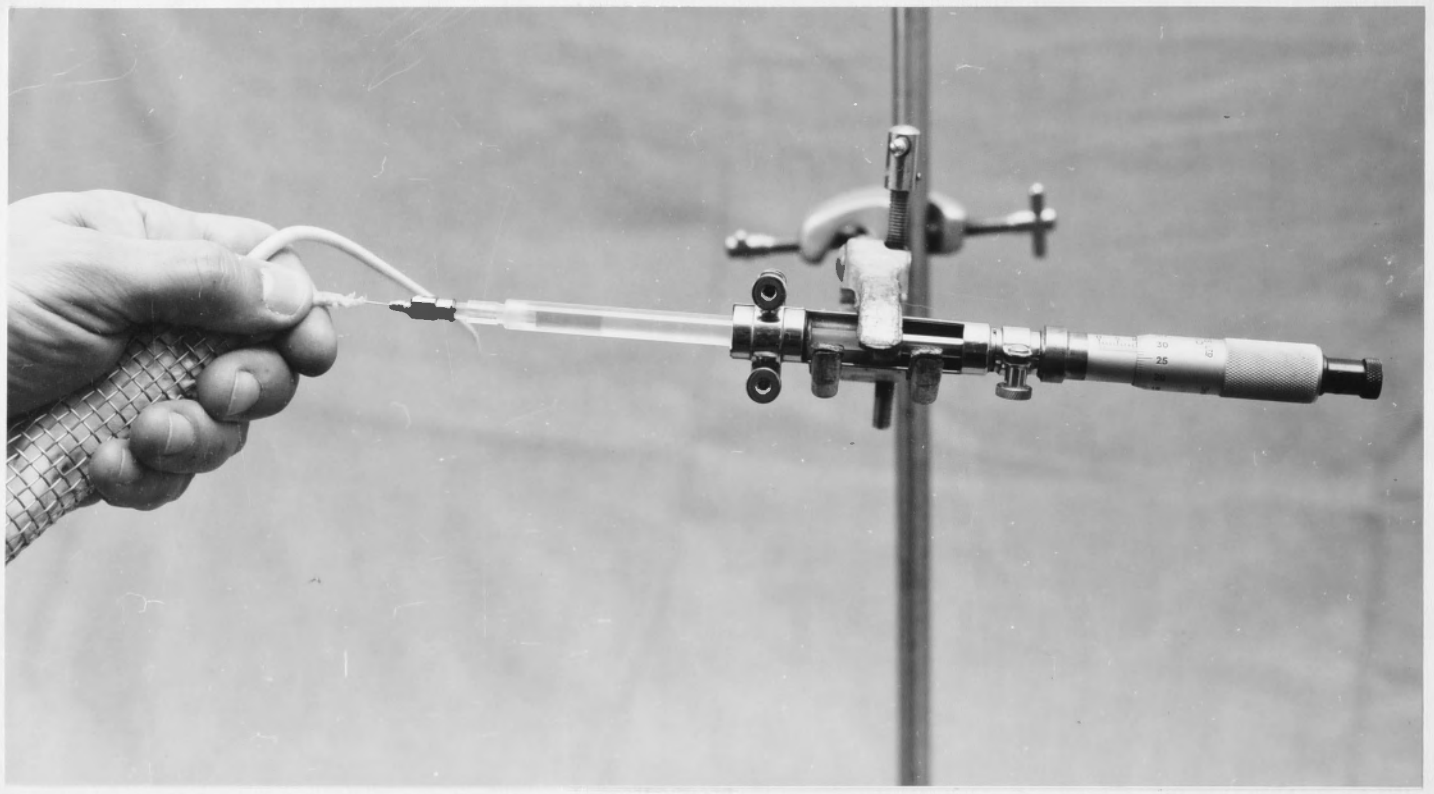

Figure 4.

The method of injection used in carrying out footpad tests.

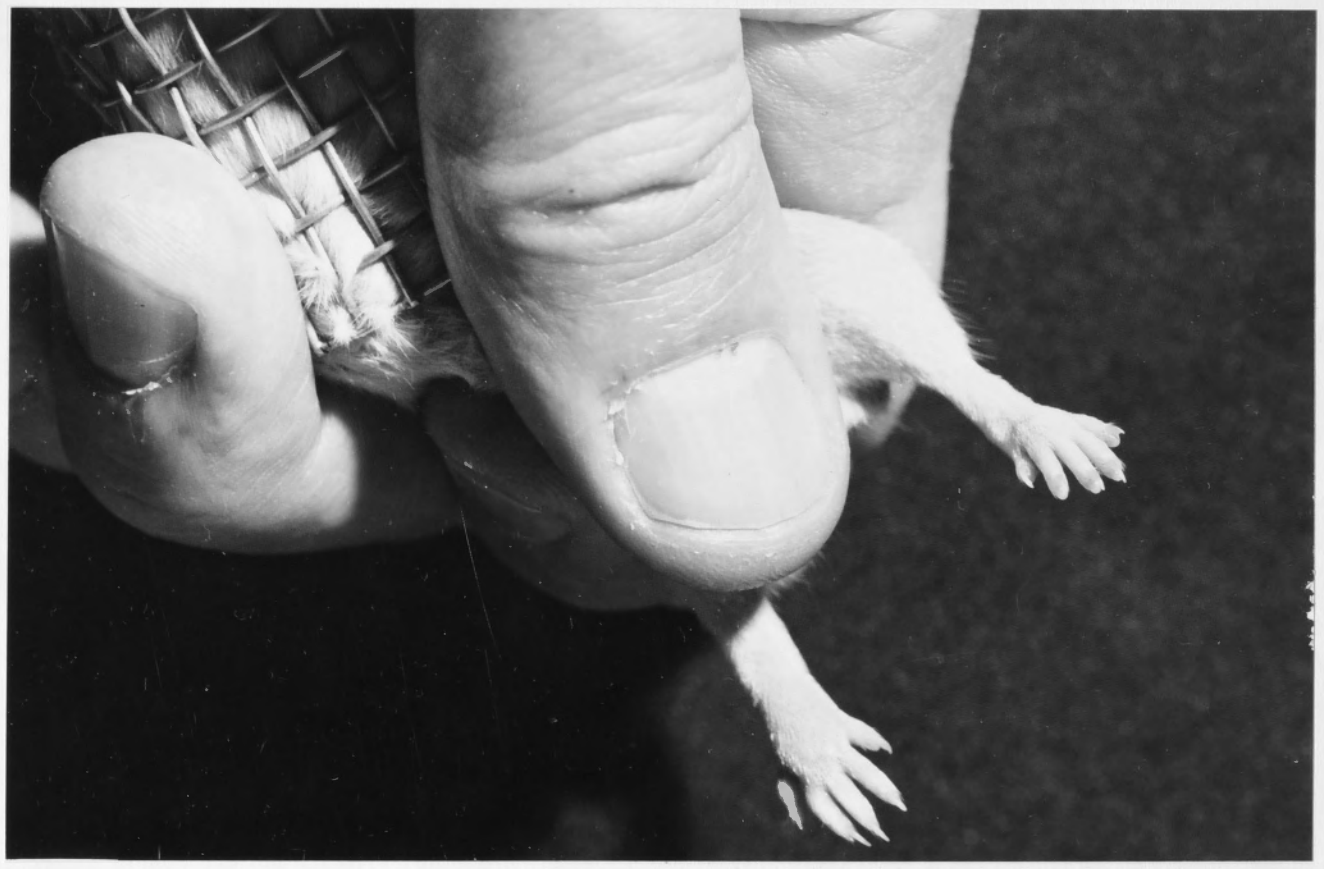

Figure 5.

A fairly marked delayed reaction - 36% increase in footpad thickness at 24 hours.

(see Figure 4). This needle is inserted into the skin of the foot just beneath one of the two apical inter-digital pads. In the earlier work the injection was subcutaneous, producing a uniform swelling of the foot, but subsequently it was found that intra-dermal injection could be carried out reproducibly. At intervals after injection, the dorso-ventral thickness of the foot is measured with dial-gauge calipers (Schnelltaster, System Kröplin, Type A.02T, H.C. Kröplin G.m.b.H., Schluchtern, Hessen, Germany), which read to 0.05 mm. These are applied in standard fashion, so that the mid-point of the circular "jaw" lies over the metatarsals and its distal rim is level with the bases of the toes. Both feet are thus measured 3-5 times and the average reading recorded to the nearest 0.1 mm. With a little practice the degree of pressure required to give reproducible readings without unduly compressing the foot is easily judged and the range of the 3-5 readings taken is usually 0.1-0.2 mm. The difference in thickness of test and control feet is then expressed as a percentage of the thickness of the control foot. Figure 5 illustrates a fairly marked delayed reaction (control foot - 2.3 mm., injected foot - 3.4 mm., % increase in footpad thickness - 35%).

In general, the number of mice in each treatment group is 20 or more, as with groups of this size differences of 7-10% in mean percentage increase in footpad thickness can usually be shown to be of statistical significance.

Validity of the Technique Adopted. A number of experiments were undertaken to assess the importance of

possible sources of error in the measurement of footpad reactions.

If the assumption is made that the right and left hind feet are of equal thickness, the labour of an experiment is much reduced, for it is not necessary to identify individual mice and record the footpad thickness before injection. To test this assumption, both hind feet of a large number of normal mice were measured. In none of the 120 pairs of readings thus obtained did one exceed the other by more than 0.1 mm. The difference in thickness between right and left feet was hence considered to be negligible.

Similar experiments showed that consecutive readings on the same animal rarely differed by more than 0.1 mm. if made on normal feet or by more than 0.2 mm. if made on the feet of reactors. (All such consecutive measurements were so carried out and recorded that the observer was not aware of the first value obtained when carrying out the second measurement).

On the basis of this work the validity of the technique of measurement adopted was considered established. Unless the contrary is stated, in the experiments to be reported, the size of the footpad reaction at any time is determined by measuring both feet, as described above, and expressing the difference between the values obtained for injected and control feet as a percentage of the thickness of the control foot, "the percentage increase in footpad thickness".

Quantitative Estimation of the Overall Error in Footpad Testing

In order to assess the total error intrinsic to the testing procedure, comprising errors in injection and in measurement, the assumption is made that delayed hypersensitivity in one subject at a given time has a definite level, an approximation to which is yielded by the process of skin-testing. On this basis, if equal doses of tuberculin are injected into the skin at two anatomically comparable sites, any difference between the subsequent readings may be ascribed to error introduced either by the technique of injection or of measurement. In an extensive series of such tests in man, Nissen-Meyer, Hougen and Edwards (1951) found the total experimental error to represent 16 to 27% of the mean diameter of induration. The size of the error was not directly related to the mean induration and the greatest percentage error occurred in the groups with the smallest means (as only six groups are recorded, the association may be fortuitous, but appears not improbable).

Similar experiments were carried out to estimate the error of measurements of hypersensitivity reactions in mice. As the only suitable anatomically comparable sites were the hind feet, the usual technique had to be modified.

Mice infected 10 days before with $2.3 \times 10^4$ units of L. monocytogenes were marked for individual identification and the thickness of each hind foot recorded. Both hind feet were then injected with 10 µl. of listerin subcutaneously. The

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TABLE 4

Comparison of reactions in right and left feet of mice infected 10 days earlier with $2.3 \times 10^4$ listeriae *

| % Increase in Thickness of: | | Difference between Values for Right and Left Feet |
|---|---|---|
| Right Foot | Left Foot | |
| 47.6 | 45.0 | + 2.6 |
| 59.1 | 72.7 | - 13.6 |
| 57.1 | 57.1 | 0 |
| 31.8 | 40.9 | - 9.1 |
| 27.3 | 27.3 | 0 |
| 36.4 | 27.3 | + 9.1 |
| 59.1 | 50.0 | + 9.1 |
| 52.4 | 38.1 | + 14.3 |
| 33.3 | 25.0 | + 8.3 |
| 38.1 | 50.0 | -11.9 |
| 17.4 | 21.7 | - 4.3 |
| 40.0 | 35.0 | + 5.0 |
| 33.3 | 42.9 | - 9.6 |
| 50.0 | 45.5 | + 4.5 |
| 54.5 | 40.9 | + 13.6 |
| 27.3 | 31.8 | - 4.5 |

*The complete protocol is not given.

TABLE 5

Estimation of the Error in Measurement of Footpad Reactions

| Group | Number of mice | Mean % Increase in Footpad thickness | | Error |
|---|---|---|---|---|
| | | Right Foot | Left Foot | |
| A | 27 | 43.0 | 39.0 | 3.1 |
| B | 33 | 38.6 | 38.5 | 5.0 |
| A + B | 60 | 41.0 | 38.8 | 4.3 |
| C | 26 | 25.7 | 22.7 | 7.0 |

thickness of each foot was again measured 24 hours later. These readings yielded two values for the intensity of the delayed reaction in each mouse, and from these the mean percentage increase in footpad thickness and the error of the group mean was calculated. In Table 4 appear a number of pairs of individual readings and Table 5 shows the mean value for each group and the error of the group mean (the calculations are described in Chapter I).

There are no significant differences between the means of the groups A, B and A + B, by either *t* or *F* test. If the error found is expressed as a percentage of the group mean, the values range between 7 and 13%. These figures may be compared with the percentage error of 16-27% obtained by Nissen-Meyer *et al*. (*op. cit*.).

In a similar experiment carried out earlier in this investigation (referred to in Table 5 as Group C) the error was 7.0, representing 27 and 31% respectively of the mean values for right and left feet. The difference in magnitude of the error in the two experiments may be partly accounted for by the increased skill of the observer in the manipulations involved. The level of hypersensitivity of the mice in Group C was considerably lower and thus the error, when expressed as a percentage, is relatively increased.

It is appropriate to comment here on the presentation of the above results. To record an increase in footpad thickness as 43.5% apparently pretends to implausible accuracy, since this figure represents measurements of 3.3 mm. and 2.3 mm,

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TABLE 6

Comparison of Mean Footpad Reaction in Groups of Mice taken from the one experiment

| Experiment and Group | Percentage increase in footpad thickness at 24 hours | | Probability+ |
|---|---|---|---|
| | Mean | Variance | |
| I* a | 25.6 | 200.2 | 0.7 > P > 0.6 |
| b | 25.0 | 172.1 | |
| II* a | 28.7 | 235.7 | 0.5 > P > 0.4 |
| b | 25.9 | 199.5 | |
| III* a | 33.5 | 224.5 | |
| b | 35.1 | 186.1 | 0.3 > P > 0.2 (b and c) |
| c | 30.6 | 222.5 | |
| IVø a | 17.6 | 91.9 | |
| b | 12.5 | 67.1 | 0.05 > P > 0.025 (a and d) |
| c | 14.8 | 89.2 | |
| d | 12.1 | 59.8 | |

+ In this column is indicated the probability that the observed difference between means is due to chance. Where an experiment contains more than two groups, the groups compared are indicated in brackets beneath.

* The infecting dose was approximately $10^4$ L. monocytogenes.

ø The infecting dose was $3 \times 10^2$ L. monocytogenes.

each of which is accurate at best to ± 0.05 mm. As the readings are subjected to one or more statistical manipulations, it was thought advisable to avoid approximations in the raw data.

Further evidence of the reproducibility of such measurements was sought by comparing the results obtained when footpad tests were carried out on two or more groups of mice of the same age and sex and infected with the same inoculum. A number of such results are presented in Table 6.

The results show clearly that mice which are as far as possible similar with respect to age, sex and conditions of infection develop the same mean level of delayed-type hypersensitivity within the limits of the method of estimation used. If the general level of reactivity is low, however, the variability of the response (see Chapter IV) is such that the chance occurrence of a few large readings in one group may increase the mean value to a point where the difference is apparently significant, as in experiment IV of Table 6. The risk is much less where most of the animals are strong reactors. (Throughout this work, unless otherwise indicated, conclusions are not based on the results of a single experiment).

Relative Merits of Subcutaneous and Intradermal Tests.

In tests for delayed-type hypersensivity it is usual to inject the antigen intradermally. It was found that with the technique described injections could be made into the dermis of the footpad in 4 cases out of 5; an injection was considered to be intradermal if a localized, blanched swelling resulted.

TABLE 7

Comparison of Subcutaneous and Intradermal Footpad Tests

| | Subcutaneous Test | | Intradermal Test | |
|---|---|---|---|---|
| | Mean | Variance | Mean | Variance |
| Experiment I | | | | |
| 3 hour-Reaction | 12.5 | 22.9 | 8.0 | 19.8 |
| 24-hour Reaction | 24.1 | 161.0 | 21.5 | 105.9 |
| Experiment II | | | | |
| 3-hour Reaction | 8.8 | 41.9 | 6.1 | 16.7 |
| 24-hour Reaction | 27.0 | 80.0 | 17.6 | 92.5 |
| Experiment III | | | | |
| 3-hour Reaction | 19.4 | 94.1 | 18.0 | 52.4 |
| 24-hour Reaction | 37.2 | 263.3 | 31.3 | 115.0 |

(To face Page 88).

Subcutaneous injection, which produced a uniform swelling of the foot, was more easily carried out. Various features of the reactions resulting from injection in each site were compared.

Footpad tests were carried out on groups of 20-25 comparable mice by either the subcutaneous or the intradermal route. The results are recorded in Table 7.

Inspection of Table 7 indicates that in general intradermal injection produces a smaller swelling at both 3 and 24 hours, but usually the readings are less scattered, as shown by the relatively smaller variance. No attempt was made to decide whether the reaction to intradermal injection was smaller because of specific or non-specific factors. As this procedure was technically more difficult, with a consequent wastage of animals, and showed no clear-cut advantage, subcutaneous injection was retained as the routine method.

In a recent report on tuberculin reactions in mice, Dietrich, Nordin and Bloch (1962) advocate the measurement of the foot in both dorso-ventral and medio-lateral dimensions and express their results in terms of the change in cross-sectional area of the foot. They do not present all the data required for the statistical evaluation of the method. In preliminary experiments I have found medio-lateral measurements much more difficult to carry out reproducibly, owing to the greater compressibility of the foot in this plane. It is likely that with this method statistically significant

results would be more difficult to obtain. I have not attempted to decide which method yields a better assessment of the level of delayed-type hypersensitivity.

In the experiments described in the remainder of this dissertation, measurements were almost invariably made both at 3 and 24 hours. The mean 3-hour readings are not reported unless they are of interest, but they were always found to be less than the mean 24-hour reading.

TABLE 8

Specific Activity of various materials in the Footpad Reaction

| Material injected | Mean % Increase in Footpad Thickness at 3 hours in: | | Probability of this difference |
|---|---|---|---|
| | Convalescent Mice | Normal Mice | |
| Listerin | 15.5 | 14.2 | n.s. |
| "Formalin-*Listeria*" $10^{10}$/ml. | 13.8 | 13.8 | |
| " $10^{8}$/ml. | 3.1 | 1.1 | <0.025 |
| "Phenol-*Listeria*" | 17.3 | 10.0 | <0.01 |
| BCG | 24.2 | 21.7 | n.s. |
| | Mean % Increase in Footpad Thickness at 24 hours in: | | |
| | Convalescent Mice | Normal Mice | |
| Listerin | 37.4 | 1.3 | <0.0001 |
| "Formalin-*Listeria*" $10^{10}$/ml. | 34.7 | 4.4 | <0.0001 |
| " $10^{8}$/ml. | 5.6 | 0.9 | <0.005 |
| "Phenol-*Listeria*" | 30.9 | 2.4 | <0.0001 |
| BCG | 13.3 | 10.7 | n.s. |

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## CHAPTER III

### MATERIALS ELICITING THE DELAYED REACTION

From the findings in other forms of delayed-type hypersensitivity, it was to be expected that a variety of preparations containing Listeria antigens would reveal cutaneous reactivity in mice recently infected with this organism. To confirm this assumption the following experiment was carried out. Mice ~~(M, 11 weeks)~~ were infected intravenously with $1.6 \times 10^4$ listeriae. Non-infected controls were set aside at the same time. Twenty-five days later footpad tests were carried out with the following materials, each being injected into the feet of convalescent and normal mice in a volume of 10 µl.:

Listerin,

Formalin-killed Listeria, suspensions containing $10^{10}$ and $10^8$ organisms/ml.,

Phenol-killed Listeria, a preparation equivalent to $10^{10}$ organisms/ml.,

Living BCG, a suspension of $10^{10}$ organisms/ml.

The resulting reactions are recorded in Table 8, and it is apparent that listerin and killed Listeria suspensions were capable of eliciting a marked delayed-type hypersensitivity reaction in the footpads of mice recently infected with L. monocytogenes. This reaction was specific, in that it did not occur in normal animals and BCG did not thus provoke mice showing marked hypersensitivity to Listeria antigens.

Listerin was chosen for routine use in the further investigations for a number of reasons. It could be prepared in large quantities and kept deep-frozen until required. The original medium, before inoculation, contained virtually no material precipitable with trichloracetic acid, and was shown to have no significant biological activity. This is indicated by the data of Table 9, in which reactions produced by dialysate medium in normal and convalescent mice are recorded, together with the results of testing mice from the same convalescent group with listerin. (These footpad tests were carried out by the <u>intradermal</u> injection of dialysate medium or listerin in a volume of 10 µl.).

TABLE 9

Specific Activity of Dialysate Medium and Listerin

| Test Material | Test Animal | Mean % Increase in Footpad Thickness at: | |
|---|---|---|---|
| | | 3 Hours | 24 Hours |
| Dialysate medium | Normal | 5.6 | 0.4 |
| " | Convalescent | 4.8 | 0.2 |
| Listerin | " | 8.0 | 21.5 |

Thus the dialysate medium before inoculation has minimal effects on the feet of both normal and convalescent mice, whereas after inoculation with <u>L. monocytogenes</u> it has

TABLE 10

Footpad Reactions of Normal and Convalescent Mice to Listerin

| Mice | Mean % Increase in Footpad Thickness at: | | | |
|---|---|---|---|---|
| | 3 hours | | 24 hours | |
| | Mean | Range | Mean | Range |
| Normal | 19.7 | 8.3 - 4.58 | 2.1 | 0 - 8.3 |
| Convalescent | 18.0 | 8.7 - 39.1 | 29.4 | 4.4 - 60.9 |

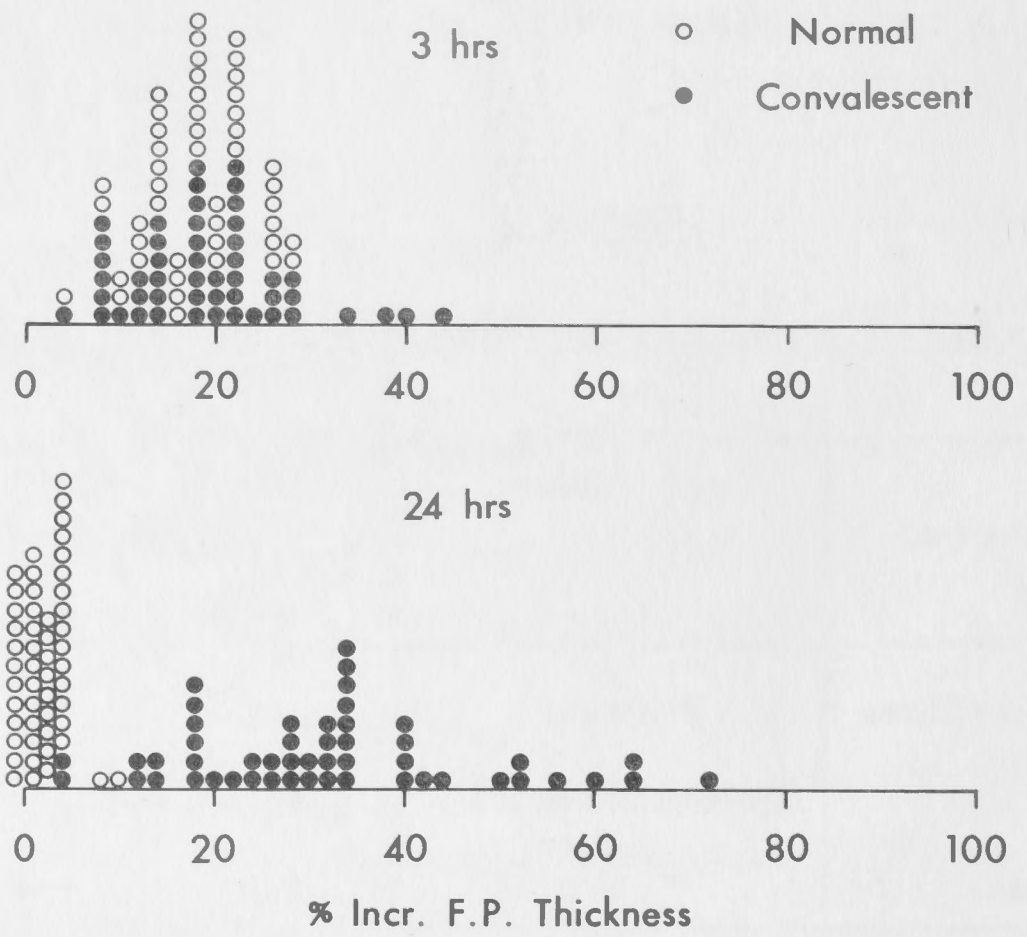


Figure 6.

Footpad reactions of individual mice 3 hours and 24 hours after the injection of 10 µl. of listerin. The convalescent mice were infected with 8 x $10^3$ listeriae 20 days earlier. (In this type of diagram the position of a symbol on the horizontal axis indicates the magnitude of an individual result; its position on the vertical axis is without significance, except that it indicates the frequency distribution).

acquired the capacity to elicit a marked delayed reaction in mice recently infected with this organism.

Effects of listerin on normal mice. As Listeria monocytogenes is known to produce a haemolysin active against the red cells of several species and also a haemosensitin (Seeliger, 1958), and has been shown to contain polysaccharides highly toxic for mice and rabbits (Stanley, 1949), the toxicity of listerin in mice was examined.

Systemic Effects.

Groups of mice were injected by the intraperitoneal route with 1 ml. of listerin and with a dose of a concentrate equivalent to 5 ml. Most looked sick within a few minutes of the injection, but recovered during the following few hours. No deaths occurred in either group in the subsequent week.

Local Effects.

In various experiments 10 µl. of listerin were injected into the footpads of normal and convalescent mice. Typical results are set out in Table 10 and Figure 6.

On the basis of the 3-hour readings the two groups of mice are indistinguishable. At 24 hours, however, mice recently infected with Listeria are differentiated from normal mice in a very clear-cut fashion, with almost no overlap. It can be seen from Figure 6 that only 2 of the 48 reactions at 24 hours were less than 10%, whereas no normal mouse showed a reaction greater than 10%.

Throughout this investigation two different batches

of listerin were used. Both were prepared under the same conditions and both had the same activity within the limits of the assay method used to compare them. The two preparations were tested in comparable groups of mice on three occasions, with results such as are shown in Table 11.

TABLE 11

Comparison of the Activity of different batches of Listerin

| Material injected | Mean % Increase in Footpad Thickness at: | |
|---|---|---|
| | 3 Hours | 24 Hours |
| Listerin I | 10.8* | 27.0 |
| Listerin II | 12.5 | 24.1 |

* There are no significant differences between either the 3-hour or the 24-hour reactions produced by the two preparations.

The work reviewed in this Chapter shows that listerin has little toxicity for normal mice and evokes a specific reaction when injected into the footpads of mice recently infected with Listeria monocytogenes.

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TABLE 12

Percentage Increase in Footpad Thickness (Mean and Range) at intervals after the injection of Listerin into the foot

| Time after Injection, Hours | % Increase in Footpad Thickness | |
|---|---|---|
| | Mean | Range |
| 3 | 10.8 | 4 - 30 |
| 6 | 19.2 | 4 - 33 |
| 12 | 29.0 | 7 - 57 |
| 18 | 33.4 | 4 - 74 |
| 24 | 39.8 | 4 - 100 |
| 30 | 38.6 | 0 - 100 |
| 42 | 29.1 | 4 - 82 |
| 48 | 27.3 | 0 - 78 |
| 54 | 17.5 | 0 - 61 |

TABLE 13

Evolution of the Footpad Reaction in Individual Mice

| Mouse | Footpad thickness at hour indicated (listerin injected at 0 hours) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3 | 6 | 12 | 18 | 24 | 30 | 48 |
| 1 | 17 | 32 | 57 | 74 | 100 | 100 | 61 |
| 2 | 18 | 22 | 45 | 45 | 50 | 50 | 35 |
| 3 | 17 | 17 | 16 | 12 | 17 | 17 | 4 |
| 4 | 13 | 26 | 26 | 31 | 35 | 35 | 22 |
| 5 | 9 | 18 | 26 | 39 | 57 | 48 | 44 |
| 6 | 5 | 9 | 18 | 14 | 24 | 18 | 18 |
| 7 | 9 | 8 | 17 | 13 | 8 | 4 | 4 |
| 8 | 4 | 4 | 8 | 12 | 8 | 8 | 0 |
| 9 | 9 | 18 | 27 | 23 | 36 | 41 | 22 |
| 10 | 30 | 30 | 44 | 52 | 65 | 63 | 52 |

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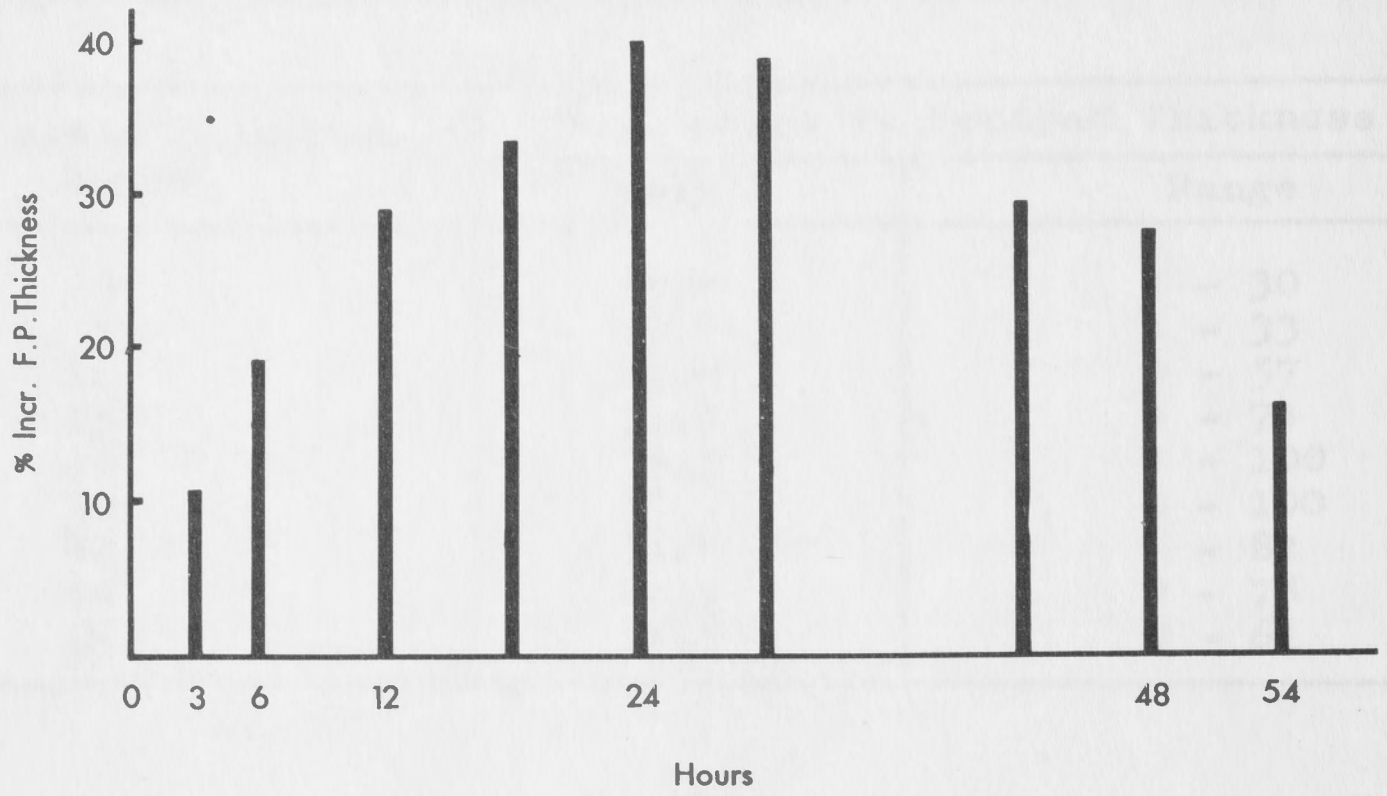


Figure 7.

Mean % increase in footpad thickness of convalescent mice at intervals after injection of listerin.

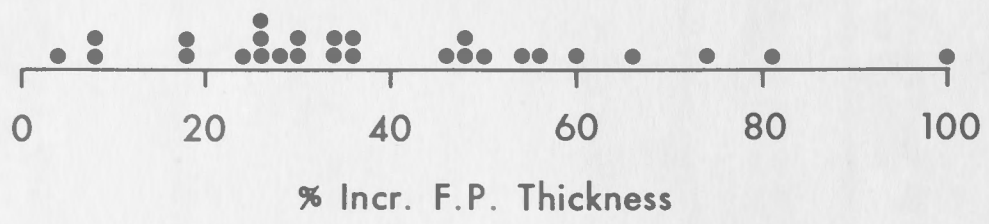


Figure 8.

Individual footpad reactions of convalescent mice 24 hours after injection of listerin.

# CHAPTER IV

## SOME ASPECTS OF THE FOOTPAD REACTION IN LISTERIOSIS

Previous work (Mackaness, 1962), already reviewed in Section IV of the Introduction, had confirmed the occurrence of delayed-type hypersensitivity in listeriosis of mice. The evolution of the reaction was now examined by means of the method described in Chapter II. In the earlier experiments 50 µl. of listerin were injected in order to elicit a footpad reaction. This volume produces a degree of mechanical distention which obscures the early phases of the reaction.

Mice were infected intravenously with $9.3 \times 10^3$ viable units of L. monocytogenes. On day 20, 10 µl. of listerin were injected into the right footpads of a group of these convalescent mice, which were marked for identification. The increase in footpad thickness was measured at intervals. Table 12 and Figure 7 indicate the mean values at each time, while in Table 13 a number of serial readings on individual mice are presented.

These results show that the footpad reaction in listeriosis follows the course characteristic of delayed-type hypersensitivity. There is little swelling at 3 hours and thenceforth there occurs a steady increase to a peak at 24-30 hours, the reaction being still well in evidence at 48 hours. This swelling is accompanied by some reddening, but necrosis has not been observed.

(To face Page 95).

TABLE 14

Footpad reactions of Animals recently infected with approximately $10^4$ listeriae (data from a number of different experiments)

| Inoculum given intravenously | Mean % Increase in Footpad Thickness at: | |
|---|---|---|
| | 3 Hours | 24 Hours |
| $8 \times 10^3$ | 17.5 | 30.6 |
| $8 \times 10^3$ | 16.0 | 35.1 |
| $1.8 \times 10^4$ | 14.3 | 30.3 |
| $1.8 \times 10^4$ | 17.7 | 27.4 |
| $9 \times 10^3$ | 19.3 | 33.4 |
| $9 \times 10^3$ | 17.9 | 37.8 |

TABLE 15

Development of Delayed-type Hypersensitivity in Inbred and Outbred Mice

| Strain | Percentage Increase in Footpad Thickness at: | | | |
|---|---|---|---|---|
| | 3 Hours | | 24 Hours | |
| | Mean | Range | Mean | Range |
| Hall | 16.1 | 8 - 26 | 24.2 | 4 - 61 |
| Bagg | 11.6 | 0 - 24 | 32.1 | 5 - 57 |
| CBA | 10.0 | 0 - 15 | 17.3 | 0 - 35 |

These mice were used in a subsequent experiment and all showed a high degree of acquired resistance, all immunity indices being greater than 19 and most over 100. All must therefore be assumed to have actually undergone infection. From Table 12 it can be seen that the range of the increase in footpad thickness at 24 hours is very wide, 4-100%. Figure 8 gives the individual 24-hour reactions. It can be seen that the distribution is unimodal, i.e. the reactions do not fall clearly into "positive" and "negative" groups.

Despite this wide individual variation, inoculation of ca $10^4$ organisms intravenously led to the development, in most cases, of a mean increase in footpad thickness of over 25%, and often almost 40%. Table 14 summarizes data from a number of experiments in which mice received an inoculum of this order and were footpad tested 3-4 weeks later.

This variability in the individual response meant that large groups had to be used in order to obtain reproducible results (in the data of Table 14, the smallest group represented is 21). Accordingly, an attempt was made to evaluate the importance of two factors possibly involved.

Hall mice and mice of two inbred strains were infected with $6.4 \times 10^3$ Listeria. The inbred mice are in general rather smaller than the outbred strain, but this variable was disregarded. Three weeks later the groups underwent footpad tests in the usual way (see Table 15).

It was found that the level of hypersensitivity

developed by Bagg mice was equal to that of the Hall mice and also that the variation in response, as reflected in the range and the variance of the 24-hour reactions, paralleled that of the Hall animals.

Another possible factor in the variability of the delayed response was the method of inoculation of bacteria. The following experiment was carried out to determine within what range the inocula received by individual mice would fall when aliquots of the same suspension were injected in the standard fashion (see Chapter I). The mice injected were later used to determine the range of individual spleen counts and the range of individual footpad reactions.

Mice were injected with an inoculum of 0.1 ml. delivered from one of four different syringes. A plate count was carried out on the bacterial suspension in the usual way and yielded the value of $1.7 \times 10^5$ organisms per ml. Immediately the injections were concluded, each of the syringes was used to eject 0.1 ml. into 19.9 ml. of medium. A volume of 0.1 ml. of this 1/200 dilution was then plated in triplicate. This process was repeated five times for each syringe. From these results, the mean number of organisms in the inoculum was computed to be $1.8 \times 10^5$, a value in remarkably good agreement with that yielded by the standard count, given above as $1.7 \times 10^5$. The one percent confidence limits were calculated to be $9 \times 10^4$ and $2.7 \times 10^5$ per ml. Thus inocula received by individual mice were unlikely to differ by more than a factor of three.

Twenty hours after inoculation, bacterial counts were done on the spleens and livers (homogenized together) of 20 mice. The mean value was $1.6 \times 10^4$ and the range $4.2 \times 10^3$ - $4.0 \times 10^4$ organisms/ml. homogenate. Other data on the growth curve of Listeria in the spleen had shown that, in animals inoculated with ca $10^4$ organisms, individual spleen counts would differ at most by a factor of about 50 at any time during the first four days of the infection.

On day 15 footpad tests were carried out on 25 of the remaining mice. The mean reaction was 36.9% and the range 4-68%.

The ratio of the maximum value to the minimum value was thus of the same order in the case of the spleen counts and footpad reactions (50 and 20, approximately) and both ratios are considerably greater than the maximum-minimum ratio for the inoculum (3). However it is unlikely that the variability of the delayed response in the individual is to be ascribed to differences in the extent to which the bacteria proliferate. In Chapter V it will be shown that a hundredfold decrease in the size of the inoculum produces only a twofold decrease in mean footpad reaction. Thus the wide variation in the delayed response manifested by individual mice is unlikely to be due to any possible inaccuracy in the inoculation procedure.

The experiments just described indicate that a greater uniformity in the footpad reactivity of Listeria-infected mice was not likely to be achieved either by the use

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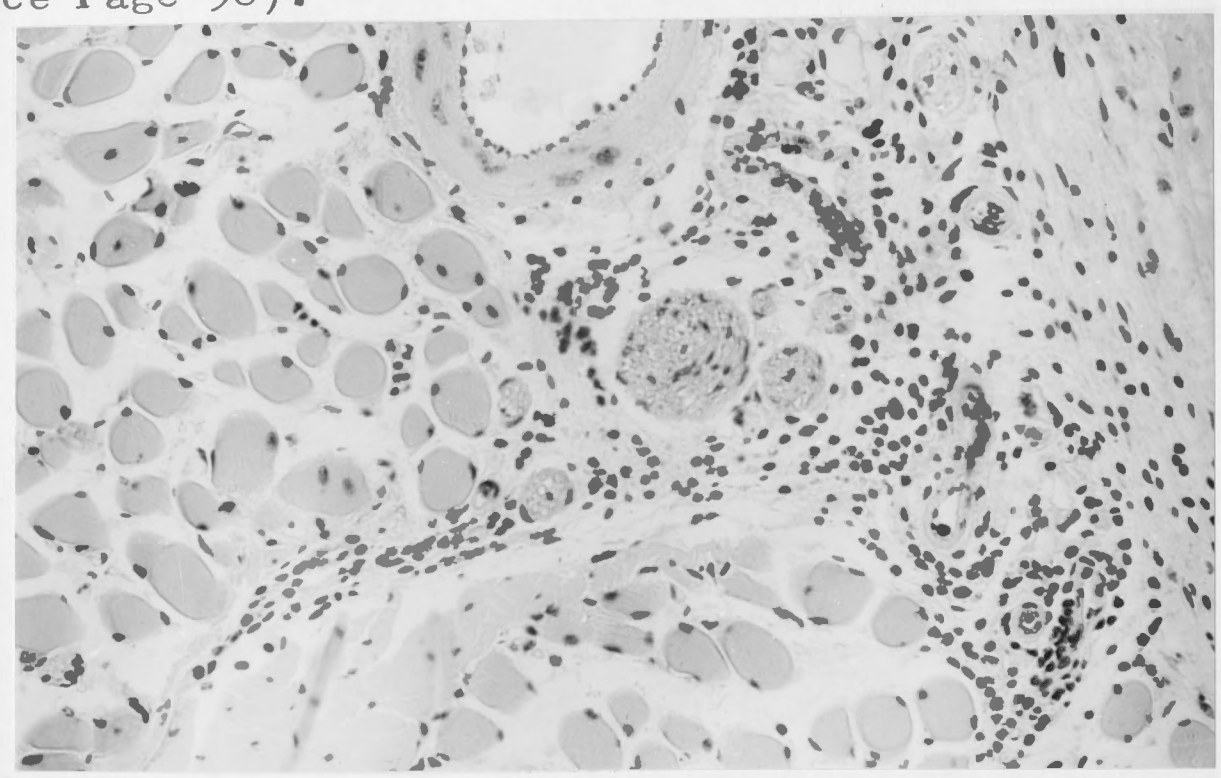

Figure 9.

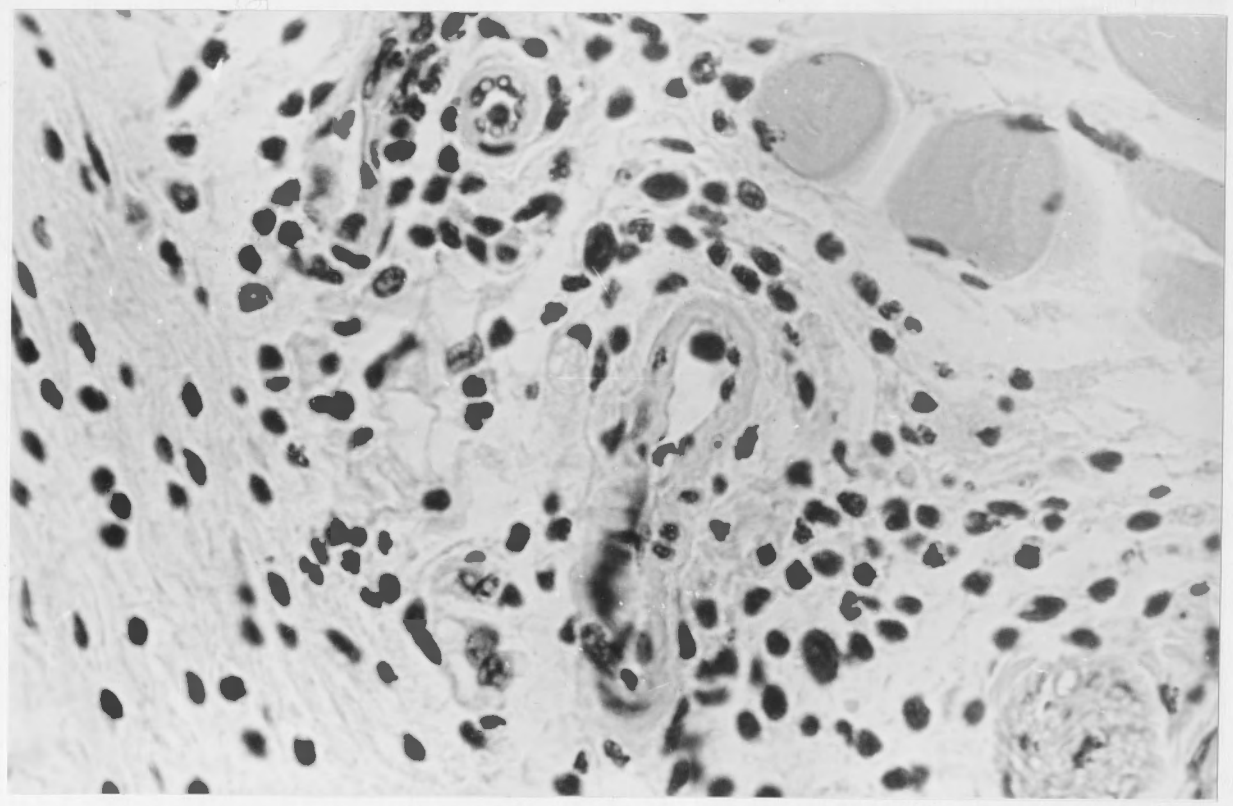

Figure 10.

Perivascular cellular infiltration at junction of subcutaneous tissue and muscle layer on sole of foot; hypersensitive mouse, 7 hours after injection of listerin. Figure 9: X 200; Figure 10: X 500. Haematoxylin and eosin.

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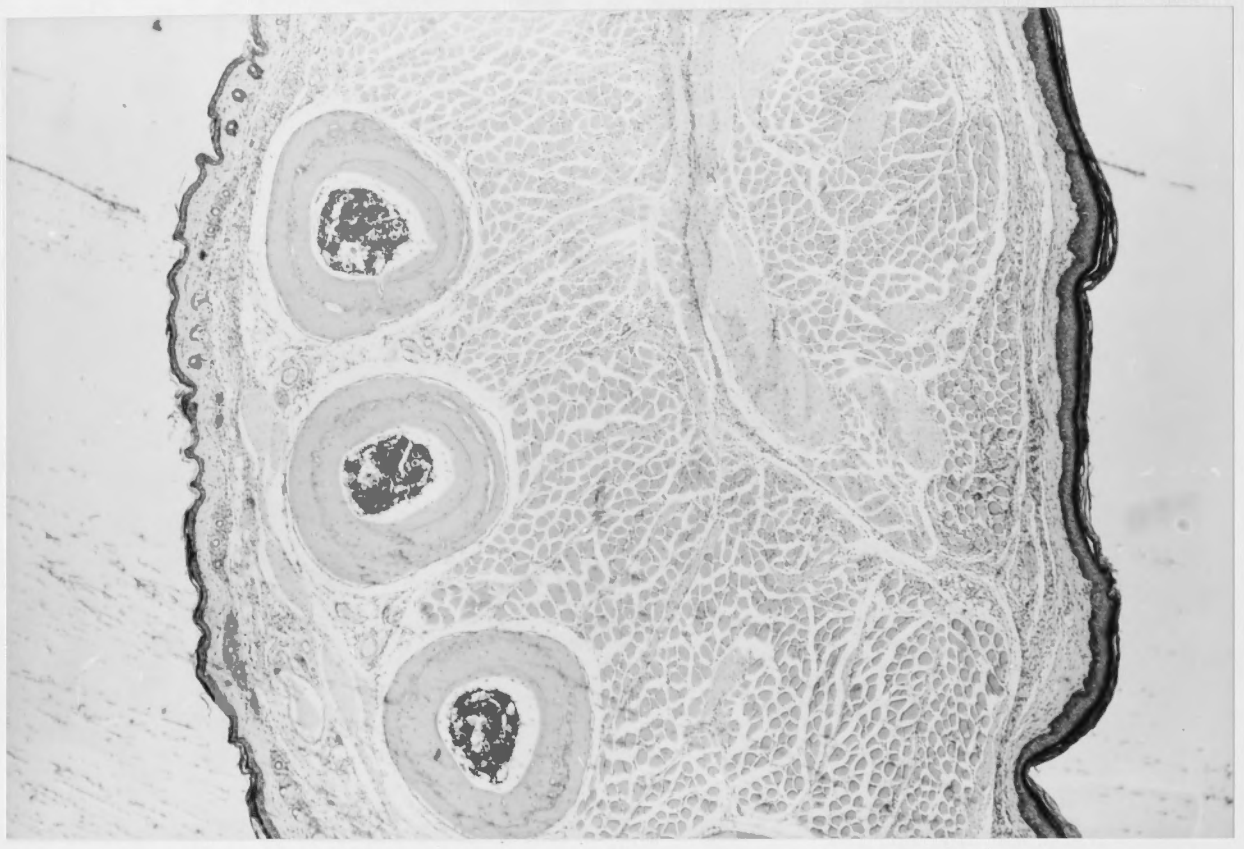

Figure 11.

Figure 12.

Transverse sections of foot of normal (Figure 11) and hypersensitive (Figure 12) mouse 24 hours after injection of listerin into the plantar surface (on right of section).
X 35. Haematoxylin and eosin.

of inbred animals or by greater refinement of the inoculation procedure. The practical consequence of this was that the size of the groups used could not be reduced below 20, if statistically significant results were to be obtained.

Histology of the Footpad Reaction. The histological features of the reaction to listerin were studied in transverse sections stained with haematoxylin and eosin. As the feet were not measured before fixation, in order to avoid compression, the degree of hypersensitivity manifested by the convalescent animals was assessed by inspection. The sections were taken from animals showing reactions estimated at 30-40%. The hypersensitive animals were infected intravenously 18 days before with $2.3 \times 10^4$ viable units of L. monocytogenes. Both normal and convalescent mice received an injection of 10 µl. of listerin in the footpad according to the standard technique.

Seven hours after this injection hypersensitive animals show accumulations of cells, mainly mononuclear, though polymorphonuclears are also present (Figures 9 and 10). At this stage cellular infiltration is most marked in the deep subcutaneous tissues adjacent to the muscle layers, and can be seen both on the plantar aspect of the foot, where the injection was made, and on the dorsum.

At twenty-four hours considerable numbers of cells are present. This is readily apparent from Figures 11 and 12, where comparison of low-power views of normal and

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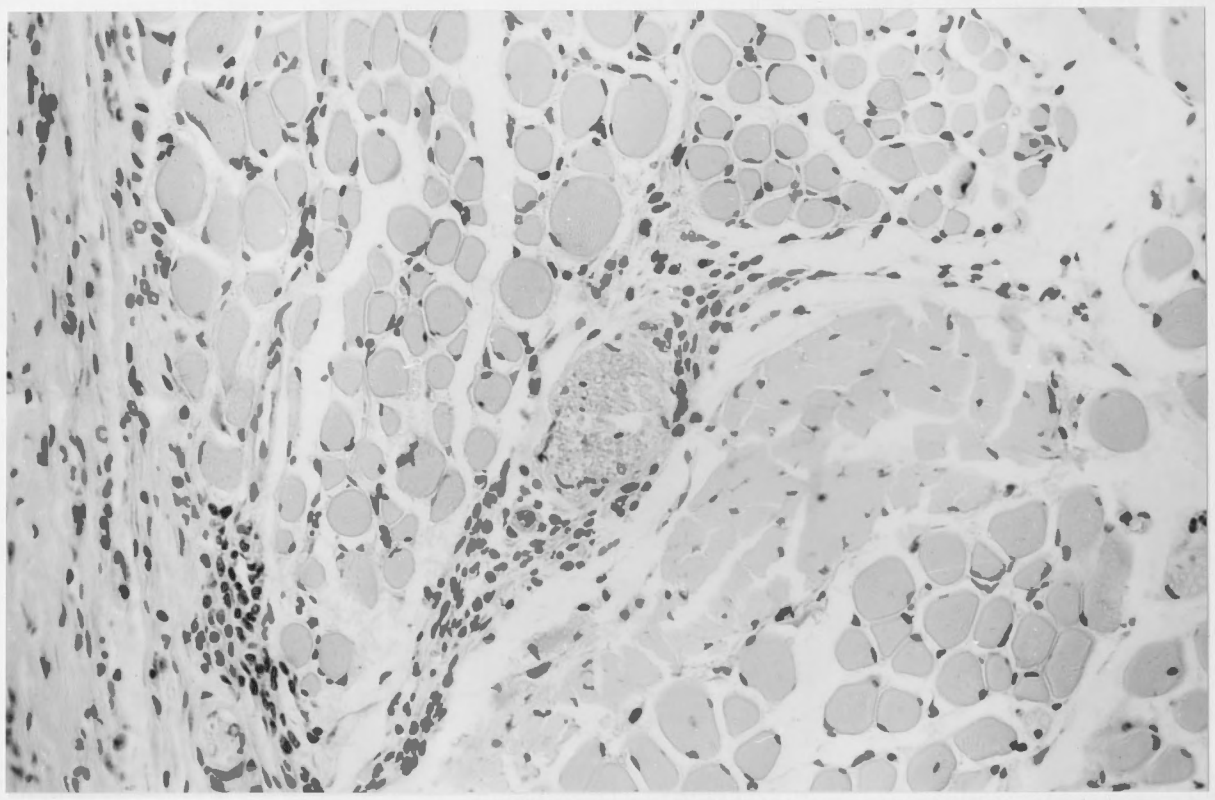

Figure 13.

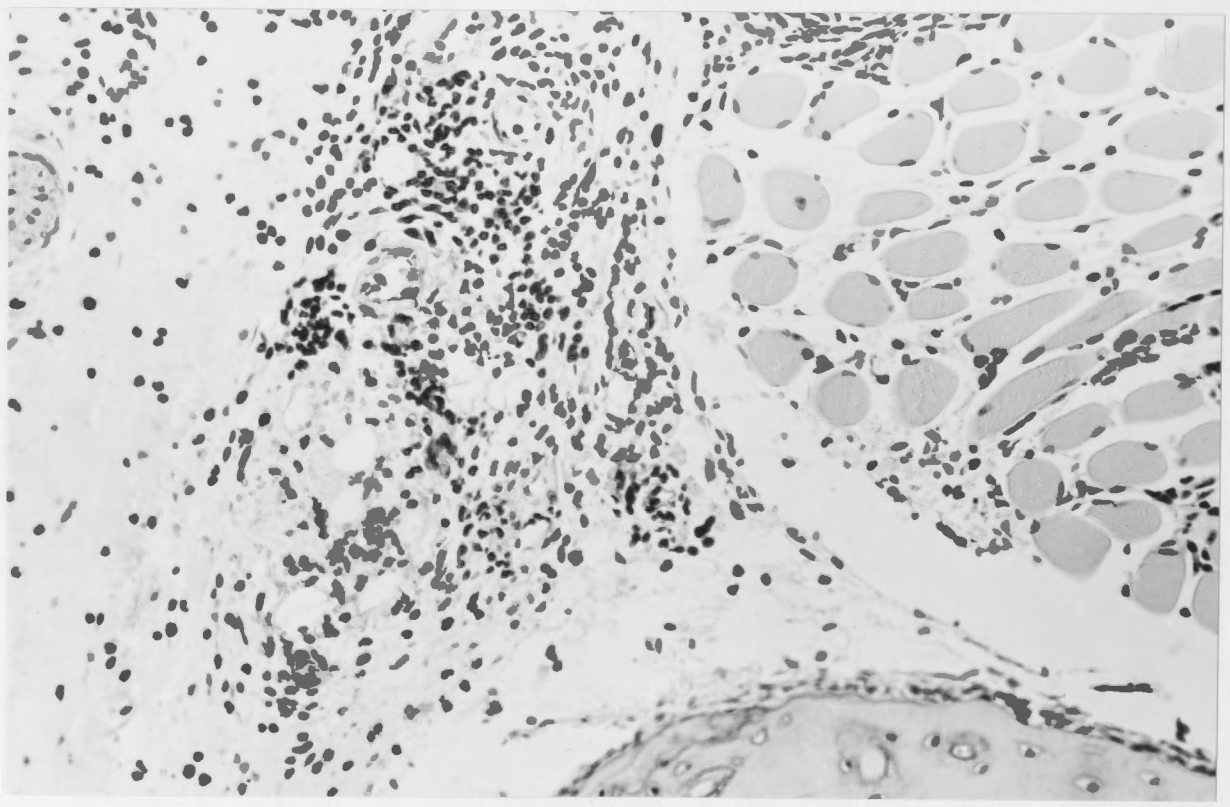

Figure 14.

Cellular infiltration in normal (Figure 13) and hypersensitive (Figure 14) tissues 24 hours after injection of listerin. X 200. Haematoxylin and eosin.

hypersensitive feet reveals a conspicuous infiltration of cells in both dorsal and plantar tissues and also along the fascial planes within the musculature. In the same sections it can be seen that oedema is present. This is most obvious on the dorsal surface, but extends throughout the foot also. This is indicated by the degree of separation of the muscle bundles in the hypersensitive foot and in the normal foot. At this stage the normal foot show little but a slight excess of cells. Notwithstanding the oedema, the degree of cellularity is comparable with that found in the tuberculin reaction of rabbits (Gell and Hinde, 1954). Figures13 and 14 show the same features at a higher magnification.

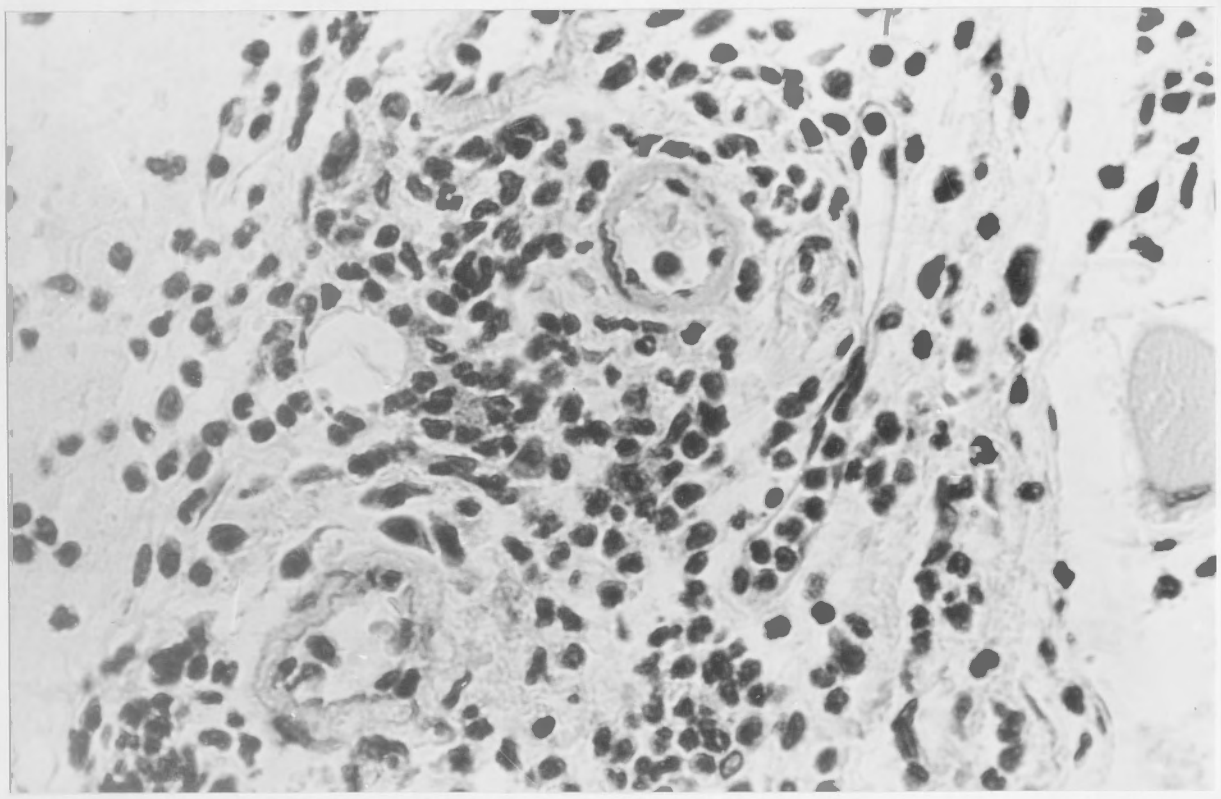

Figure 15.

Perivascular infiltrate in 24-hour reaction (hypersensitive animal). X 500. Haematoxylin and eosin.

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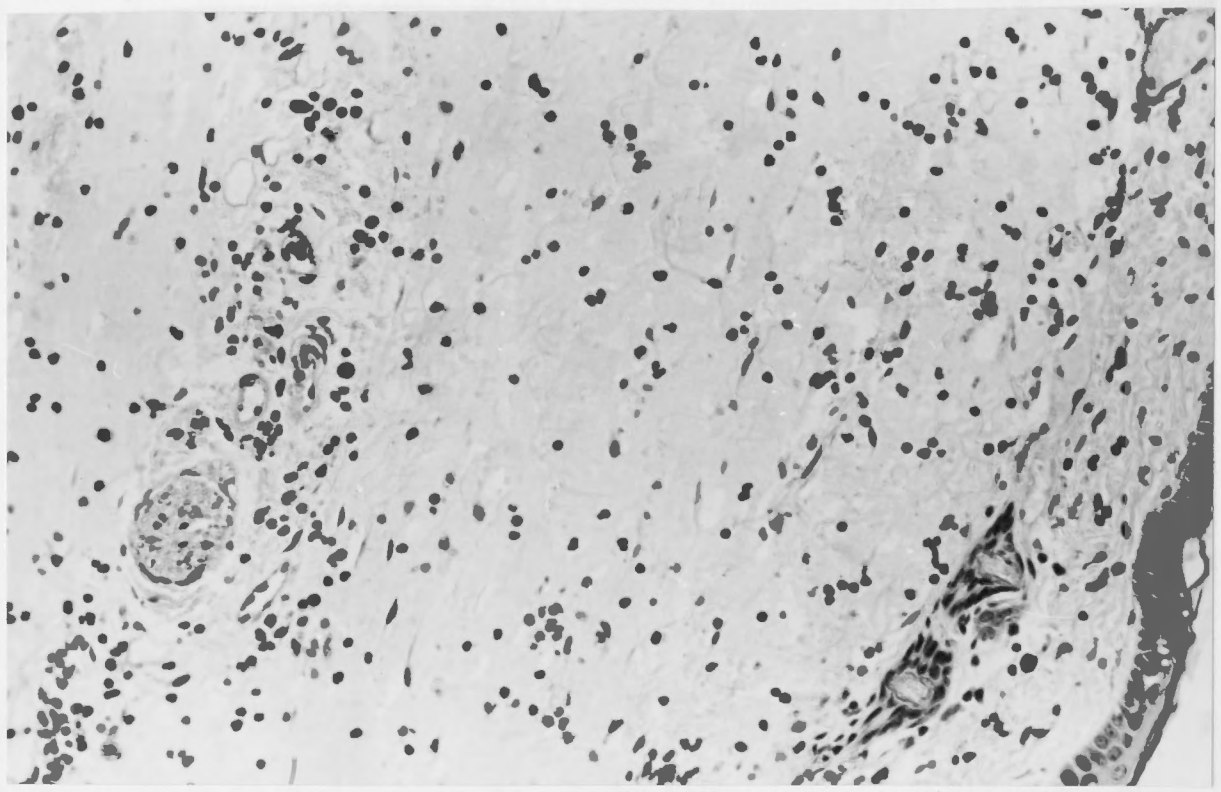

Figure 16.

Hypersensitive animal, 24 hours after injection of listerin. Cellular infiltration and oedema of dorsal tissues. X 200. Haematoxylin and eosin.

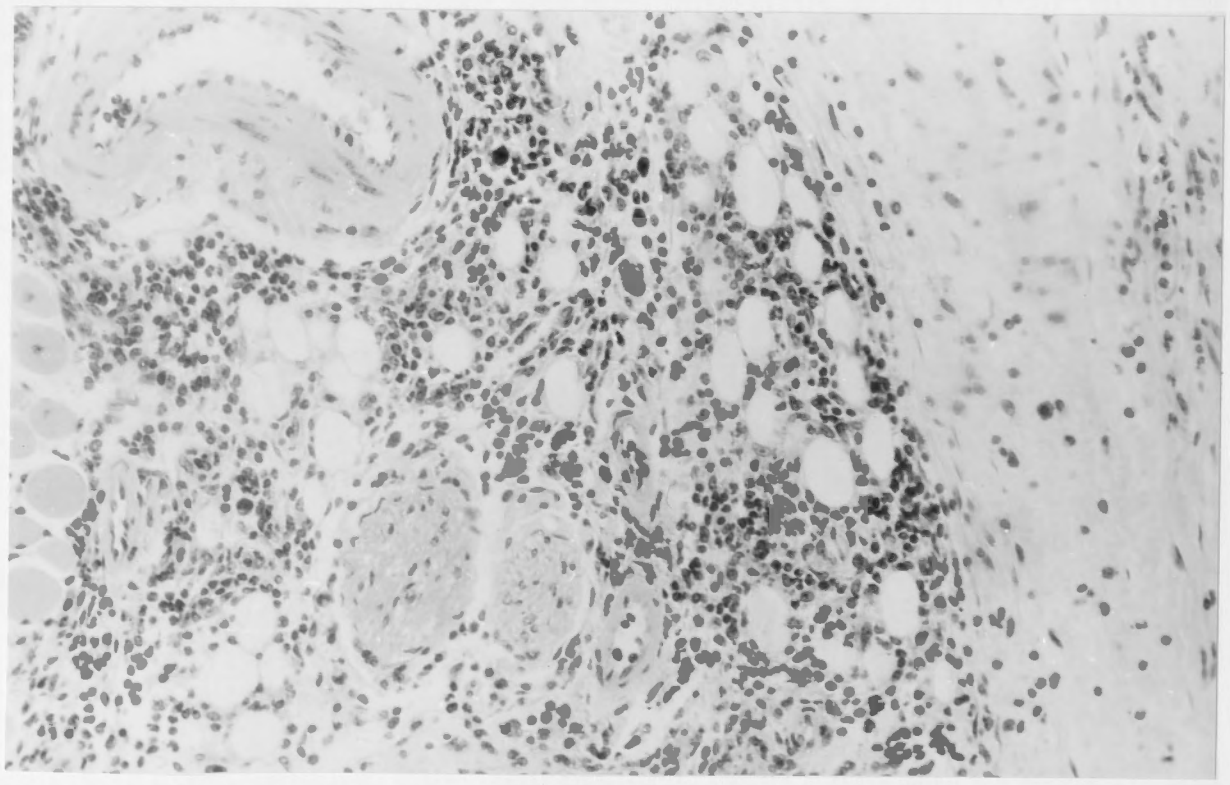

Figure 17.

Hypersensitive animal, 24 hours after injection of listerin. Cellular reaction in tissues on sole of foot. X 200. Haematoxylin and eosin.

The predominant cells in these aggregations (Figure 15) are mononuclear cells (macrophages and histiocytes), lymphocytes and, to a lesser extent, granulocytes being also represented. In Figures 16 and 17 the reactions on the dorsal and plantar surfaces of the foot are illustrated.

In a number of these sections what appears to be a small thrombosed vessel can be seen. The anatomical situation of these lesions usually suggests an origin in a vascular structure. In Figure 18 the vena comitans of a small artery seems to have been affected and here the remains of the vessel wall can be made out. As such lesions are encountered in the 24-hour reactions of both normal and hypersensitive animals, they are tentatively ascribed to a toxic effect of listerin.

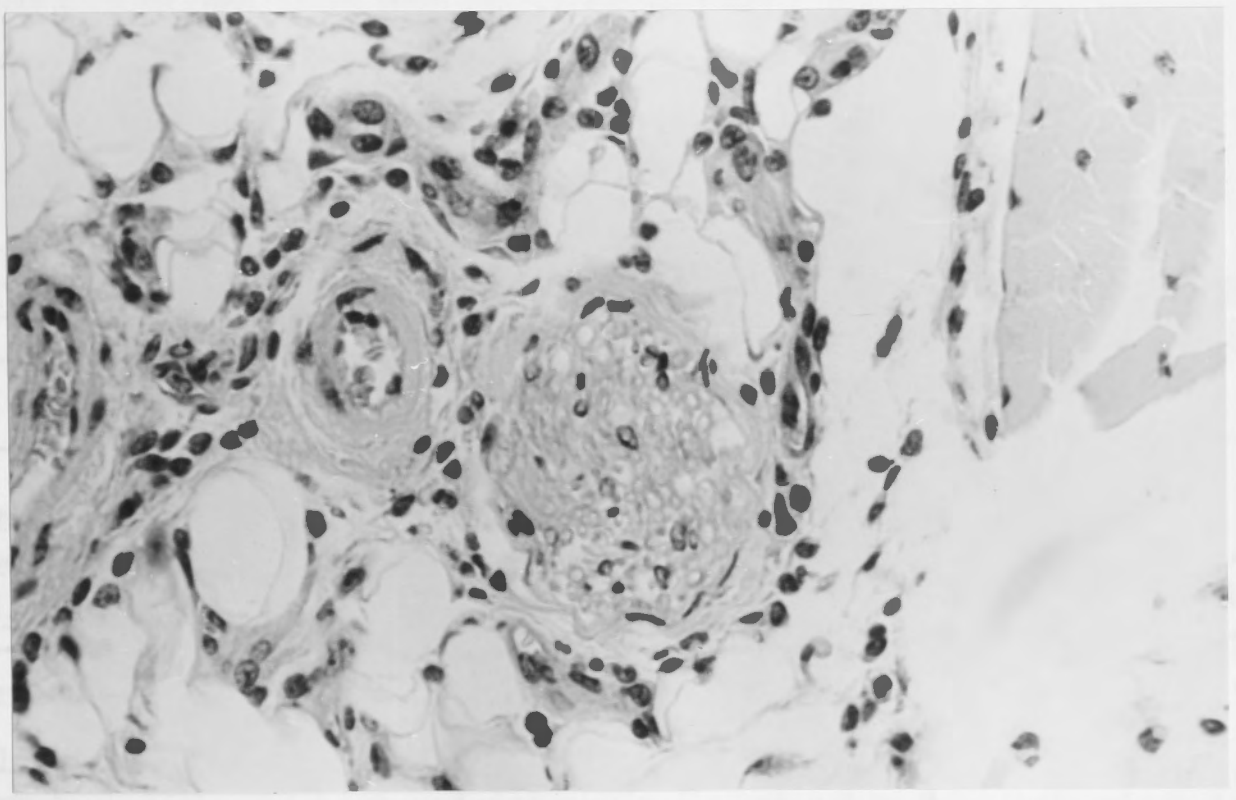

Figure 18.

Normal mouse, 24 hours after injection of listerin into the footpad. The vena comitans appears to be thrombosed. Haematoxylin and eosin.

## Induction of Delayed-type Hypersensitivity

Boyden's demonstration that immunization with tuberculoprotein depressed the response to a subsequent BCG infection in guinea-pigs has been discussed in Section II.D. of the Introduction. The following experiment was carried out to detect this phenomenon in listeriosis.

Mice were sorted into groups which, over a period of 4 weeks, received 5 injections of 0.1 ml. of one of the following materials, the first injection being given subcutaneously and the later ones intraperitoneally: listerin, a formalin-killed suspension of *Listeria* ($10^{10}$ organisms/ml.) and balanced salt solution. Two weeks after the course of injections ended all animals were infected with $6.6 \times 10^4$ listeriae. Three weeks later footpad tests were carried out on the survivors; the results are presented in Table 16.

TABLE 16

Effect of previous injections of Listeria antigens on the level of delayed sensitivity after infection

| Injections given before inoculation with *Listeria* | Mean % Increase in Footpad Thickness at: | |
|---|---|---|
| | 3 Hours | 24 Hours |
| Listerin | 23.1 | 19.8 |
| Formalin-killed *Listeria* | 19.3 | 34.0 |
| B.S.S. | 23.8 | 30.8 |

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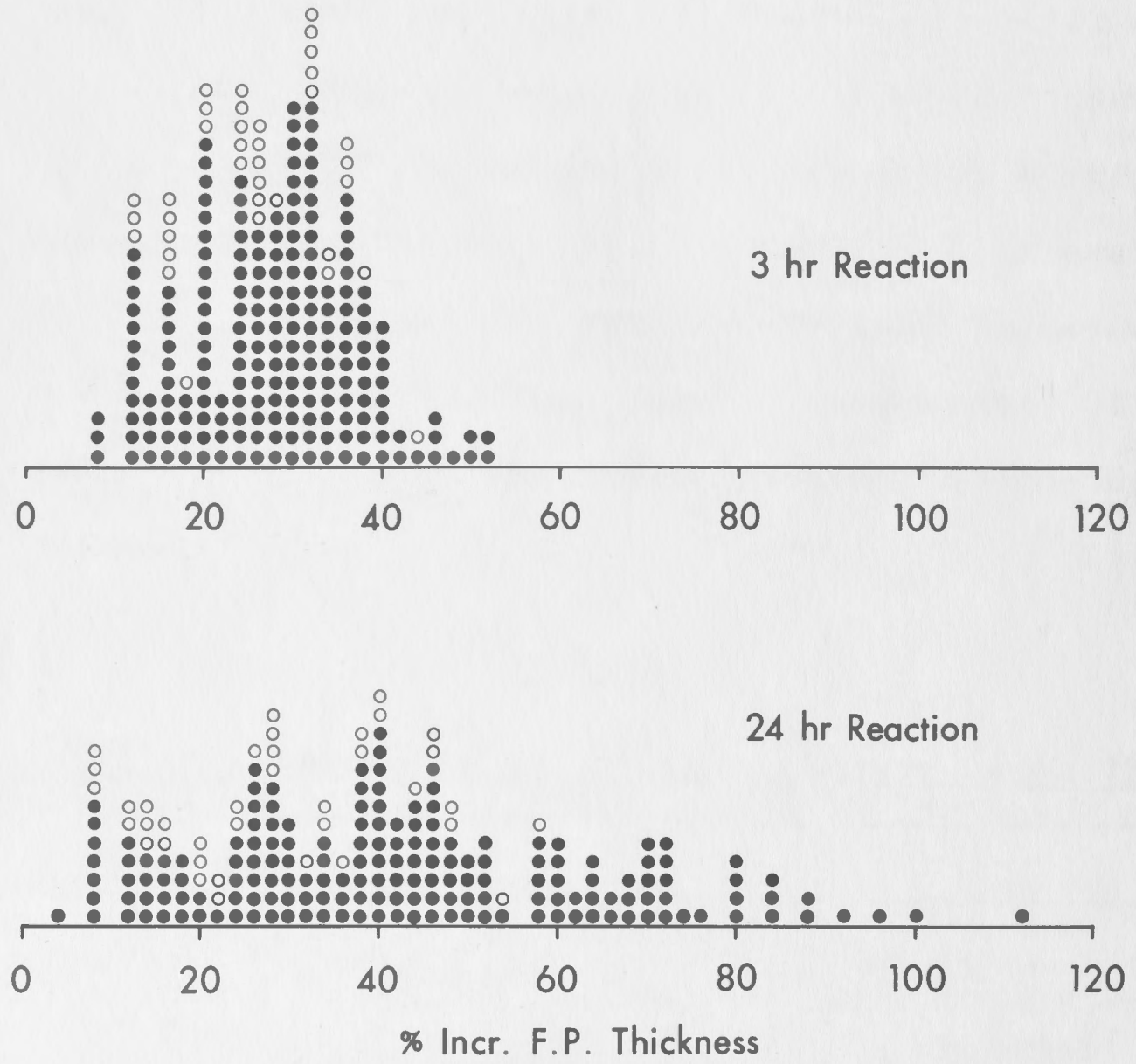


Figure 19.

Individual footpad reactions of mice injected with Listeria antigens or B.S.S. in Freund's incomplete adjuvant before infection with L. monocytogenes.

The difference in the levels of delayed-type hypersensitivity shown by the groups injected with listerin and with B.S.S. is highly significant ($P < 0.001$), thus confirming the observations of Boyden. It is surprising that killed organisms produced no such effect, as the preparations used were approximately equivalent in their ability to elicit the reaction (see Table 8). It is possible that the efficacy of a formalin-killed suspension of Listeria in eliciting a reaction in the footpad is due in large measure to the particulate nature of the material, a characteristic which would enhance its retention at the injection-site.

In order to decide whether this effect was related to the titre of circulating antibody, an attempt was made to furnish mice with high and minimal levels of antibody to listerin, by injecting a small dose of listerin intraperitoneally and a larger dose combined with incomplete adjuvant subcutaneously. Footpad tests after infection showed, however, that the reactions of the group injected with listerin-adjuvant were enhanced rather than depressed. A further experiment was then set up.

Mice received subcutaneous injections of a preparation of Listeria antigens (see Chapter I), mixed with Freund's incomplete adjuvant. Controls were given Hanks' B.S.S. in incomplete adjuvant. On day 24 all mice received $1.5 \times 10^4$ listeriae intravenously. On day 42 mice were marked for identification and footpad tests were carried out. The results are summarized in Table 17, and the individual readings appear in Figure 19. The difference between the 3-hour

TABLE 17

Effect of Injection of Listeria Antigens in Freund's Incomplete Adjuvant on the level of delayed sensitivity after infection

| Material injected in incomplete adjuvant (before infection) | Mean % Increase in Footpad Thickness at: | |
|---|---|---|
| | 3 Hours | 24 Hours |
| *Listeria* antigens | 27.7 | 43.3 |
| B.S.S. | 24.6 | 27.9 |

readings is not significant ($0.1 > P > 0,05$), that between the 24-hour readings is highly so ($P < 0.001$).

It appeared therefore that pre-treatment with *Listeria* antigens had in some way augmented the response to infection as judged by the footpad reactivity. The mechanism at work here was now investigated.

The effect of the presence of a depot of adjuvant in the body of a mouse undergoing a generalized *Listeria* infection has not been analysed in detail. On one previous occasion mice that had received Hanks B.S.S. in complete adjuvant were infected with *Listeria* along with normal mice. The subsequent 24-hour reactions were 26.7 and 34.9% respectively ($0.02 > P > 0.01$). On the basis of these results and in view of the fact that the level of delayed sensitivity in the mice injected with B.S.S. in incomplete adjuvant is not very great (see Table 14) although within "normal" limits, it may well be that there was some depression of the delayed response in the control group of the experiment recorded in

Table 17. Nonetheless, the 24-hour reactions in the group injected with Listeria antigens were the largest ever obtained, and it was therefore important to decide whether the augmented response was due to delayed-type hypersensitivity or to some other form of reactivity, possibly of Arthus type.

Histological Features. The delayed reactions of mice treated with Listeria antigens in incomplete adjuvant before infection with the organism were examined in transverse sections of the foot 24 hours after injection of 10 µl. listerin. The size of the footpad reaction was measured before the foot was removed.

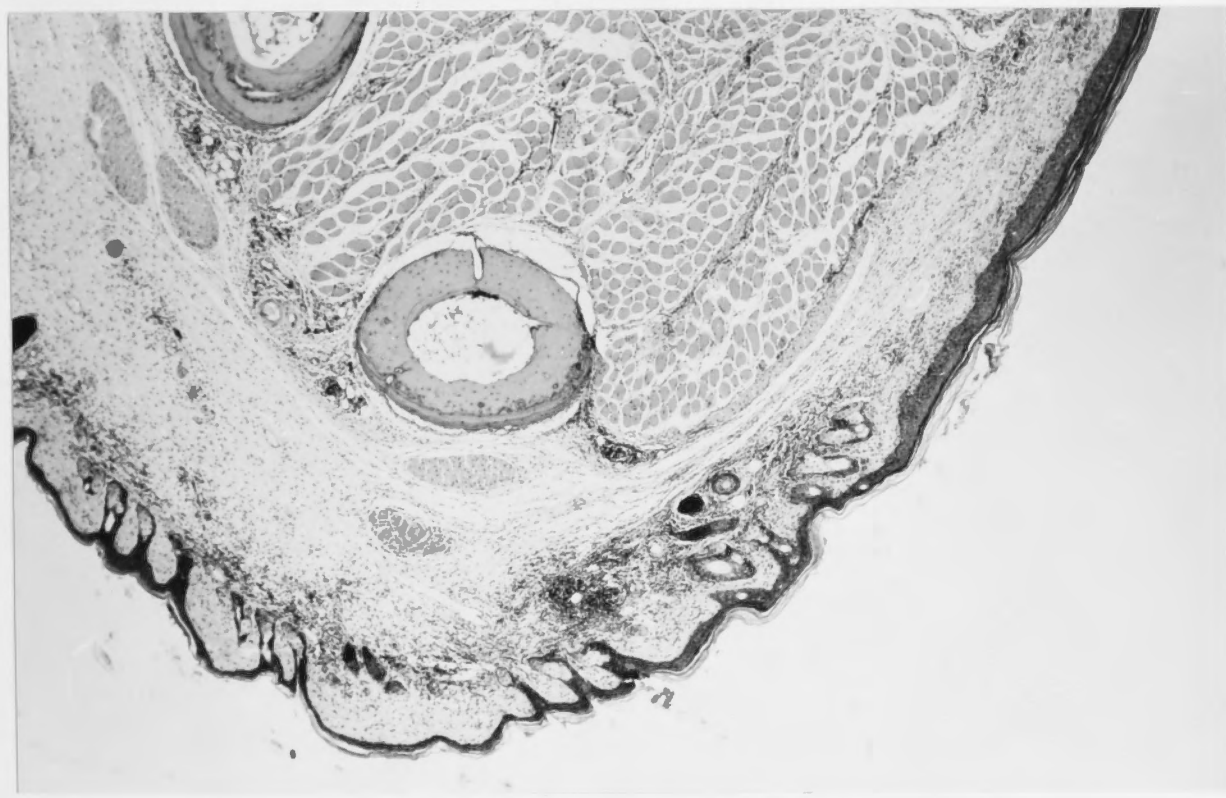

Figure 20.

Transverse section of foot (68% increase in footpad thickness at 24 hours). X 35. Haematoxylin and eosin.
All the sections illustrated in Figures 20 to 26 were taken from mice treated with Listeria antigens in incomplete adjuvant before intravenous infection with Listeria. The footpad reactions were examined 24 hours after the injection of 10 µl. of listerin according to the standard technique. The reactions were measured immediately before the feet were removed for fixation.

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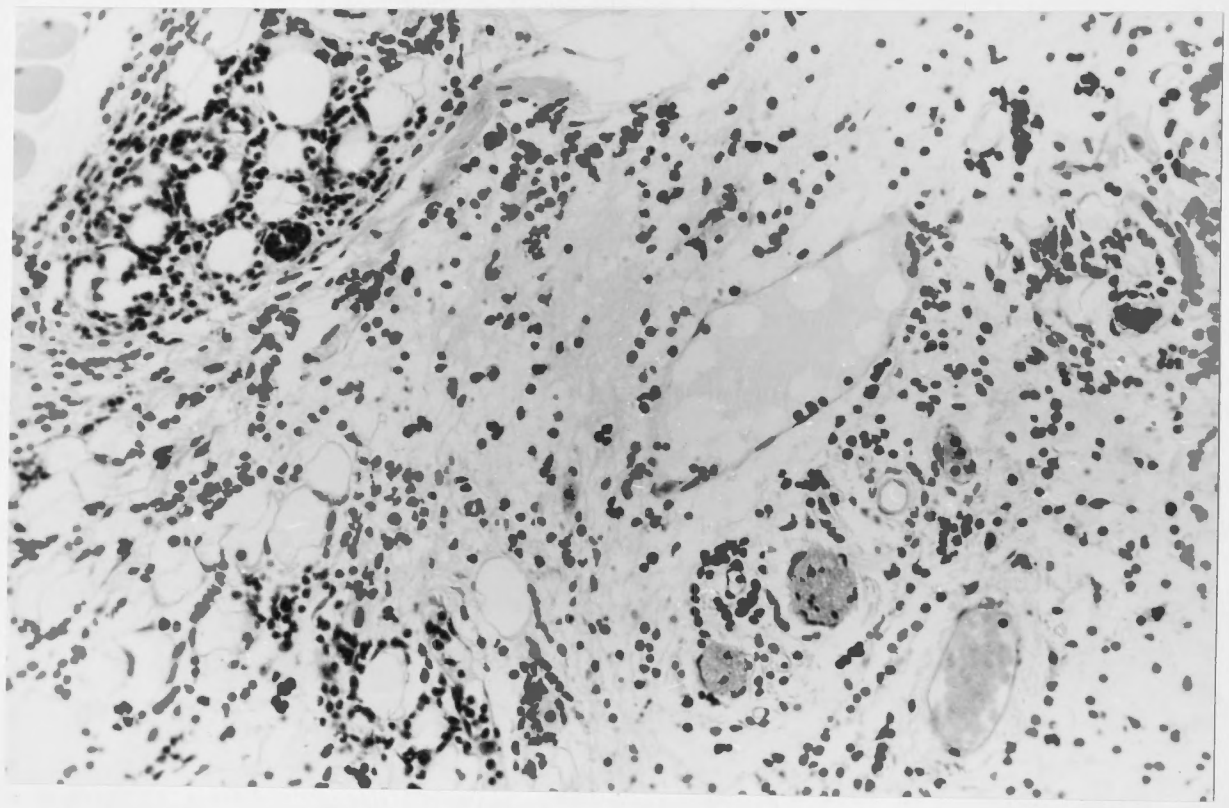

Figure 21.

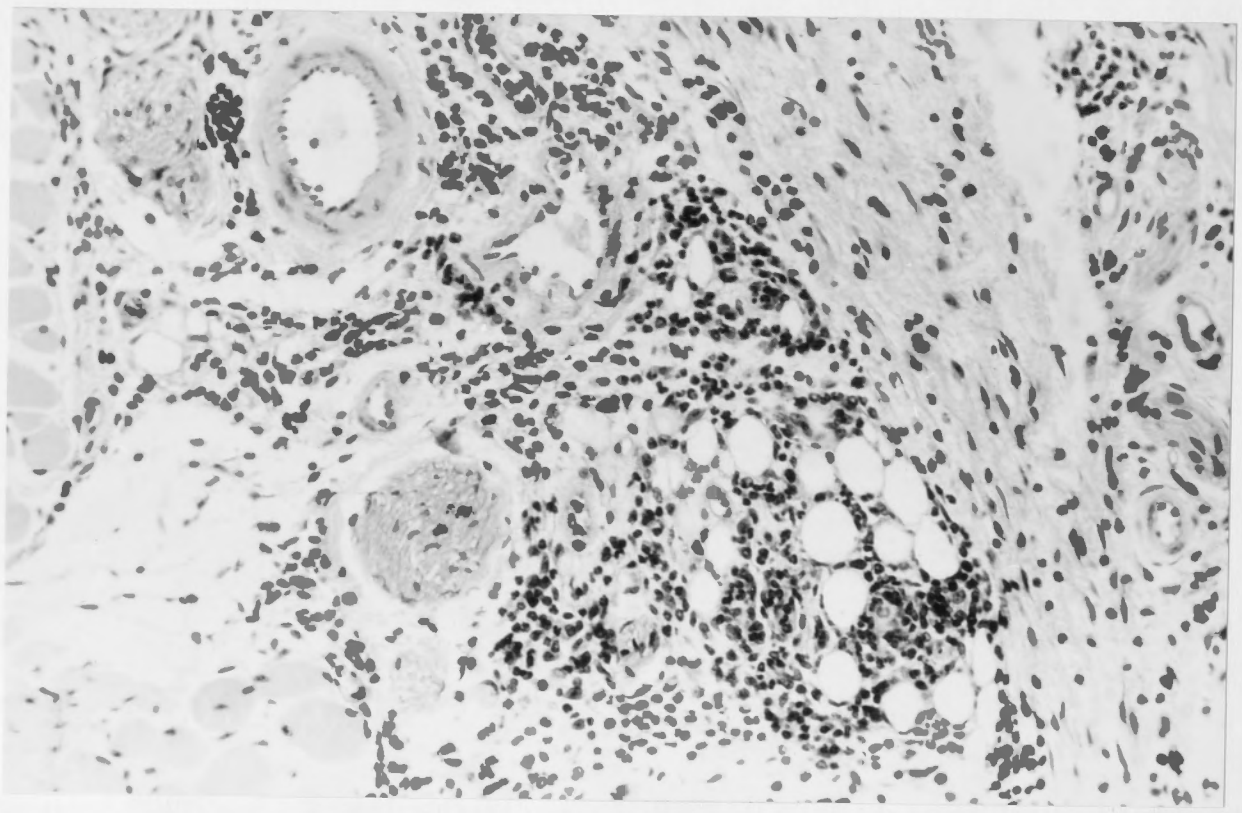

Figure 22.

Increase in footpad thickness at 24 hours - 38%.
Figure 21. Cellular infiltration and oedema in the subcutaneous tissues of the dorsal surface of the foot. X 200.
Figure 22. Cellular reaction in sole of foot. X 200.

The overall appearance in a low-power view (Figure 20) shows some oedema and marked cellular infiltration, as in the delayed reaction after simple infection (see pp. 98-100). Comparison of Figures 21 and 22 with Figures 14, 16 and 17 shows that the degree of cellularity is much the same in reactions elicited after infection or after treatment with Listeria antigens followed by infection. (As the footpad reactions were not measured in the former case, it is not certain that the two groups are quite comparable, but the measurements obtained before the sections of Figures 21 and 22 were prepared are in the range within which those of Figures 14, 16 and 17 were estimated to lie). Again some oedema is seen most obviously on the dorsum of the foot; on both aspects of the foot a marked influx of cells has taken place.

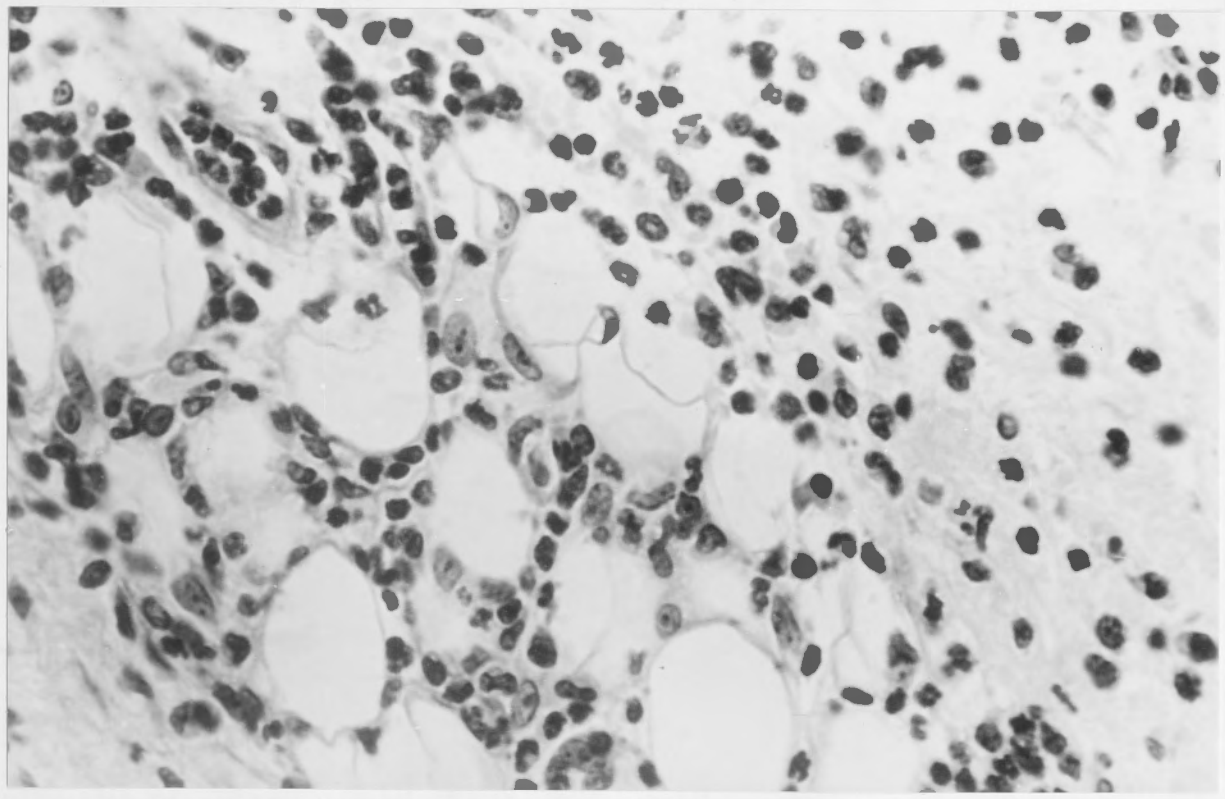

Figure 23.

Increase in footpad thickness at 24 hours - 38%.
Subcutaneous tissues of sole of foot. X 500.
Haematoxylin and eosin.

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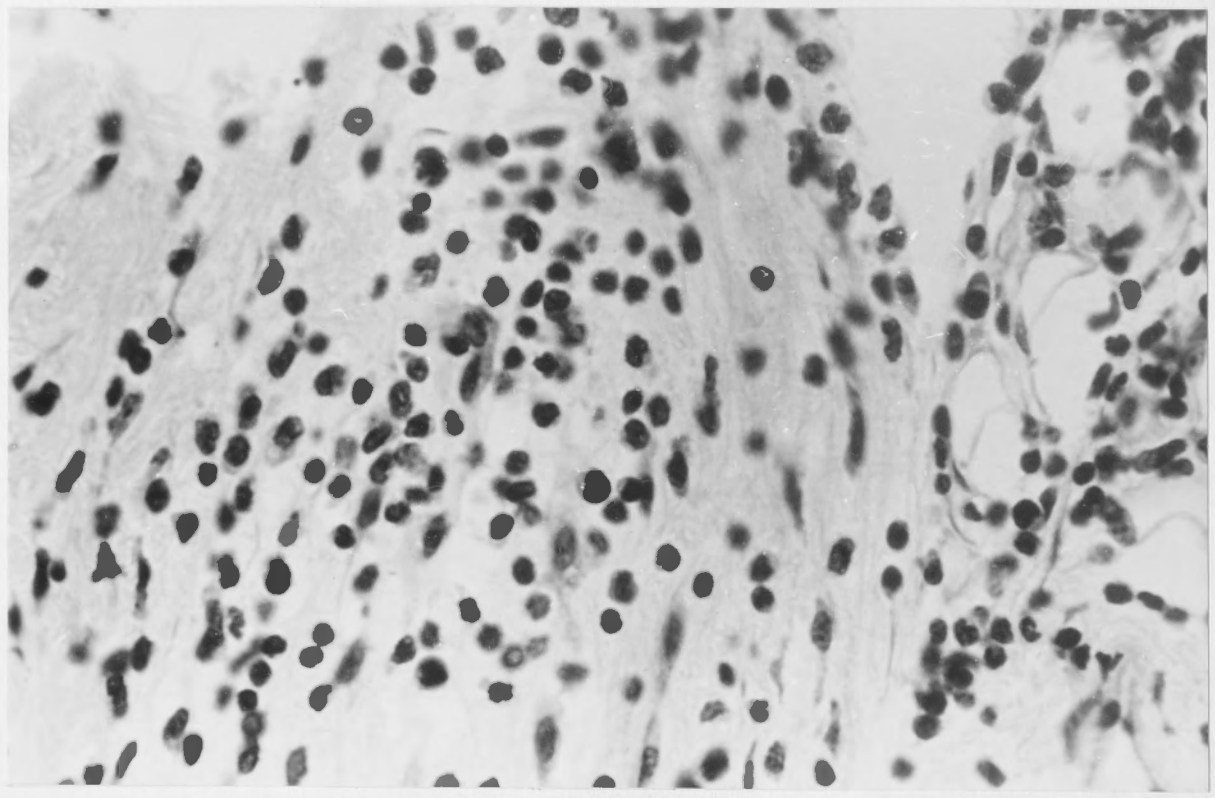

Figure 24.

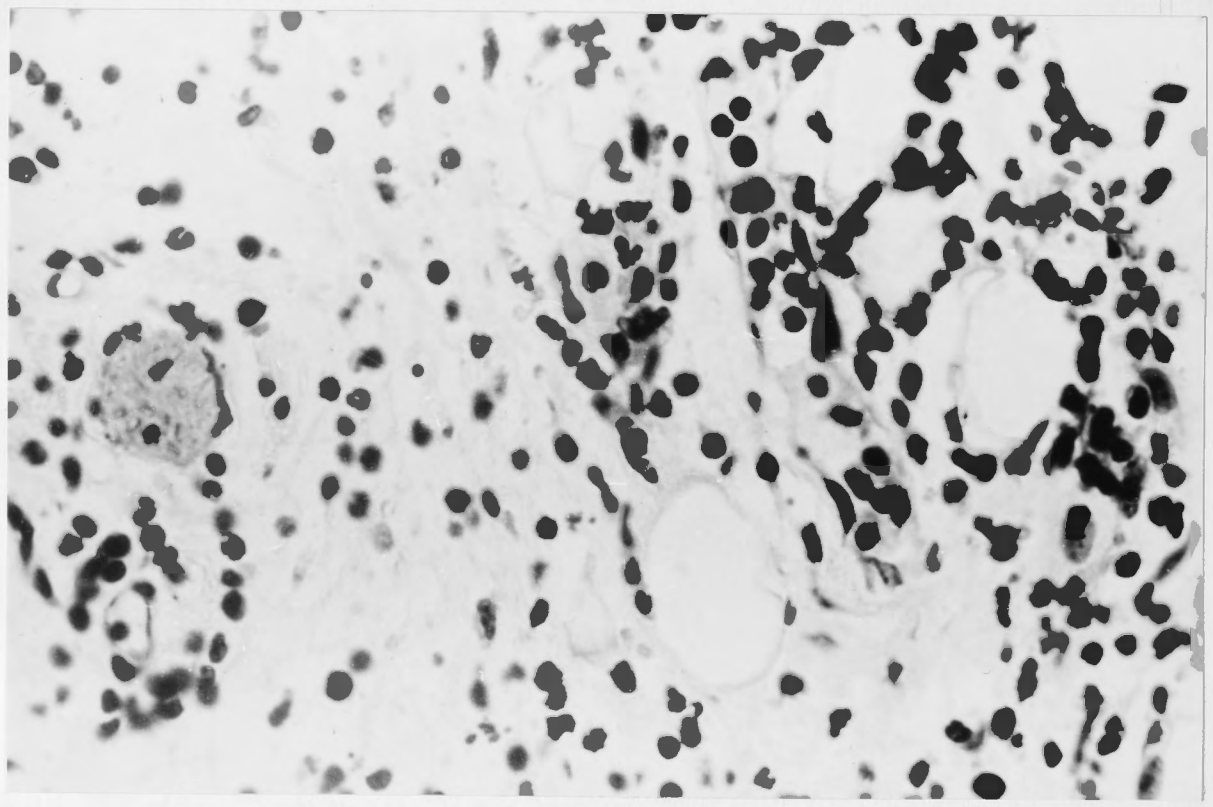

Figure 25.

Increase in footpad thickness at 24 hours - 46%.
Cellular reaction in subcutaneous tissue of plantar (Figure 24) and of dorsal (Figure 25) surface of foot. X 500.
Haematoxylin and eosin.

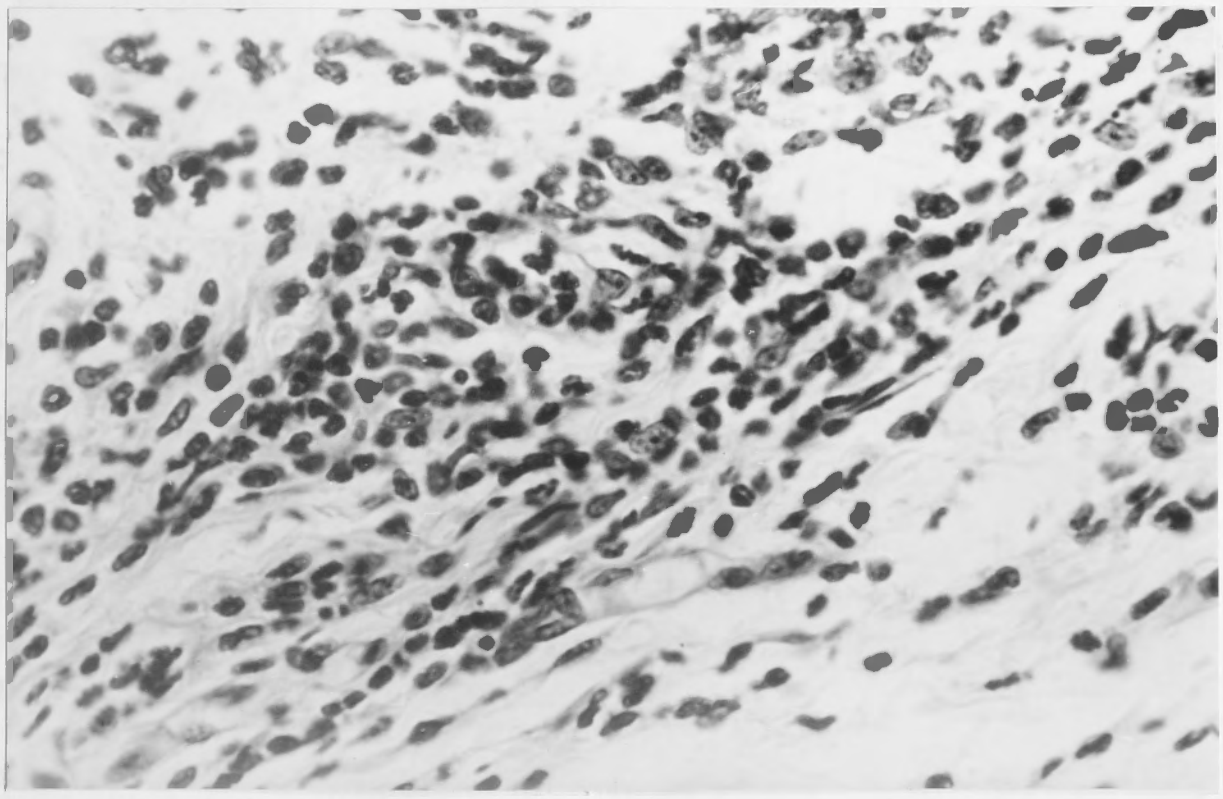

Figure 26.

Increase in footpad thickness at 24 hours - 68%.
Cellular reaction in subcutaneous tissues of sole of foot. X 500. Haematoxylin and eosin.

Mononuclear cells predominate (Figures 23 to 26); while the numbers of polymorphonuclear leucocytes are somewhat greater than in the delayed reaction after simple infection, there are few pyknotic cells and little nuclear debris, the presence of which is a marked feature of 24-hour Arthus reactions in the rabbit (Gell and Hinde, 1954).

Thus the 24-hour reaction in the feet of mice which show an augmented response to Listeria infection after injection of Listeria antigens in incomplete adjuvant is distinguished at the histological level only by the presence of polymorphonuclear leucocytes in numbers slightly greater than are found in the delayed reaction of simple infection. In both cases the majority of the cells are mononuclear. At the

site of injection both reactions show cellular infiltration and some oedema. On the plantar aspect of the foot and in the connective tissue of the muscle bundles aggregations of round cells are found in abundance in mice sensitized by either method. On morphological grounds therefore the augmented reaction is considered to be of delayed type.

If the effect were related to the presence of antibody such as is involved in the Arthus reaction, it might be expected that the size of the 24-hour reaction would be correlated with that of the 3-hour reaction. Statistical analysis showed that in fact a correlation did exist ($\underline{r}$ = 0.501, $P < 0.001$). Further analyses revealed, however, that a similar correlation could be found in some, but not all, sets of data on mice infected in the usual way, e.g. in the results presented (in part) in Table 13, where the correlation coefficient was found to be 0.56.

One group of mice in the experiment of Table 17 underwent a footpad test before infection. Here there was no correlation between 3-hour and 24-hour reactions ($\underline{r}$ = 0.02); the mean % increase in footpad thickness at 3 hours was 28.8% and at 24 hours 8.6%. Thus the factor or factors involved in this correlation were introduced by the infection and/or its interaction with processes initiated by the listerin-adjuvant depot.

If, from the individual results summarized in Table 17, one selects for analysis those pairs of readings which show either a definite rise or a definite fall between 3 hours

and 24 hours (i.e. a difference of 0.2 mm. between the two values), one finds that in each group the correlation between the 3-hour and 24-hour reactions is highly significant (Table 18).

TABLE 18

Correlation between 3-hour and 24-hour Reactions in Mice showing a clear-cut rise or fall in footpad thickness between 3 and 24 hours

| | Group I 3-hour reaction less than 24-hour reaction | Group II 3-hour reaction greater than 24-hour reaction |
|---|---|---|
| Mean 3-hour reaction | 29.3 | 28.1 |
| Mean 24-hour reaction | 55.2 | 20.6 |
| Correlation between 3-hr. and 24-hr. reactions, $r$ = | 0.655 ($P < 0.01$) | 0.747 ($P < 0.01$) |

Thus in both groups the size of the 24-hour reaction is directly related to that of the 3-hour reaction. The mean 3-hour reactions of both groups do not differ significantly. If the 3-hour reaction in each group is due to the same factor or factors, it is difficult to see why opposed effects should ensue in the two groups.

Boyden (1957) found in animals possessing both Arthus sensitivity to ovalbumin and delayed sensitivity to

TABLE 19

Effect on the footpad Reaction to Listerin of a passively induced Arthus reaction occurring simultaneously at the same site.

| Group | Subcutaneous Injection | Footpad test material | Mean Reaction 3 Hours | Mean Reaction 24 Hours | r* |
|---|---|---|---|---|---|
| A | HSA 10 mg. | listerin + anti-HSA | 34.5 | 24.0 | 0.308 |
| B | HSA 1 mg. | listerin + anti-HSA | 33.7 | 23.9 | 0.369 |
| C | HSA 10 mg. | listerin + normal serum | 26.8 | 33.7 | 0.417 |
| D | HSA 10 mg. | listerin + saline | 19.5 | 31.0 | 0.424 |
| E | Nil | listerin | 26.8 | 37.3 | 0.212 |

* r = the correlation coefficient for the relationship between the 3-hour and 24-hour reactions; the values of this statistic found for groups A to D are significant at the 5% level, while that for group E is not significant.

The data of groups A and B can be pooled, as the means and variances are virtually identical (the variances are 183.5 and 182.9 respectively), and the value of r then obtained is significant well beyond the 1% level.

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tuberculin that the tuberculin reaction was unaffected by the occurrence of an Arthus reaction at the same site. In mice this proved not to be true.

Mice convalescent from Listeria infection were divided into five groups. Each group received a subcutaneous injection of human serum albumin or B.S.S. and the following day footpad tests were performed, each of the materials listed below being injected in the volume indicated:

| | |
|---|---|
| Listerin | 10 µl. |
| Listerin + rabbit anti-HSA | 20 µl. |
| Listerin + normal rabbit serum | 20 µl. |
| Listerin + saline | 20 µl. |

Listerin was mixed in equal volumes with the other materials (serum or saline), so that the same quantity of the former reagent was injected into each animal. The scheme of injection and the results are indicated in Table 19.

The 3-hour and 24-hour reactions of groups A and B both differed significantly from those of groups C and E ($P < 0.001$ and $< 0.005$, respectively). In the case of group D it is assumed that dilution with saline facilitates the escape of the antigen from the site, since both 3-hour and 24-hour reactions were depressed.

It is thus clear that in mice the occurrence of an antigen-antibody reaction at the test site will not *per se* enhance the expression of delayed-type hypersensitivity, but will rather impede it. However the correlation between the

3-hour and 24-hour reactions is the same in the presence or absence of a specific antigen-antibody reaction. This conclusion differs from that of Boyden (1957); the contradiction is unexplained, but perhaps species differences may be invoked. The two experiments are not identical in principle, as Arthus sensitivity was induced actively by Boyden and passively in the work just described.

The mouse is stated to develop only a low degree of Arthus hypersensitivity (Chase, 1956). In fact the only reports claiming extensive Arthus reactions in mice are based on experiments in which the animals were sensitized by means of <u>complete</u> adjuvant (Freund and Stone, 1956; Benedict and Tips, 1954). The latter authors state that marked reactions were almost invariably haemorrhagic; they showed also that mouse serum was active in a reversedArthus reaction, but in low degree only.

Hence, in the mouse, severe 24-hour reactions which are cellular and non-haemorrhagic are unlikely to be due to Arthus hypersensitivity. Further support for this contention is provided by the following experiment.

As the lesions of tuberculosis and silicosis are very similar, and as the nature of the cellular response has been held to determine the type of sensitivity developed (see Section II.E. of the Introduction), the effect of silicic acid on sensitization to a protein was examined. Mice received an injection of human serum albumin in incomplete adjuvant and in incomplete adjuvant to which a suspension of

silicic acid had been added to a concentration of 4% by volume of the final albumin-adjuvant mixture. Five weeks later 100 µg. of HSA in 10 µl. was injected into the footpad and the foot measured as usual. The readings appear in Table 20.

TABLE 20

The sensitization of mice to human serum albumin in Fruend's Incomplete Adjuvant. The effect of the addition of silicic acid to the adjuvant

| Mice sensitized with HSA in: | Mean % Increase in footpad thickness at: | | |
|---|---|---|---|
| | 3 Hours | 24 Hours | 48 Hours |
| Incomplete adjuvant | 28.8 | 5.0 | 2.5 |
| Incomplete adjuvant + silicic acid | 55.6 | 18.8 | 5.6 |

It is clear that a state of delayed-type hypersensitivity was not induced, the temporal evolution of the reactions of both groups indicating rather Arthus hypersensitivity. The enormous 3-hour reaction in the animals treated with silicic acid subsides rapidly, being only one third the size at 24 hours and hardly detectable at 48 hours. While the 3-hour reactions in the group sensitized with the silicic acid and adjuvant mixture were of a somewhat darker red than usual, no necrosis was evident. (The adjuvant effect of intravenous injections of silica before

antigenic stimulation has been previously reported; the work is reviewed by Vigliani and Pernis, 1963). In other words, under conditions where antibody production was apparently at a very high level, there was no evidence of a skin reaction which could be taken as of delayed type.

It was therefore concluded that the augmented response to listeric infection found in mice previously injected with a preparation of Listeria antigens in incomplete adjuvant was chiefly a manifestation of delayed-type hypersensitivity.

# CHAPTER V.

## DELAYED-TYPE HYPERSENSITIVITY AND ACQUIRED RESISTANCE

In the three chapters to follow, findings are presented which bear directly on the central problem of this investigation, the relation between delayed-type hypersensitivity and acquired resistance. The first approach is essentially statistical: these two reactivities are examined in a number of situations which may be thought of as arising out of natural infection and in which experimental manipulation has been confined to varying the dose or the route by which infection is induced or the stage at which the variables are estimated. In this type of experiment only the reactions of groups are considered.

Later an attempt will be made to determine the relation between these two immunological responses in the individual animal.

Finally, procedures which depress or ablate the delayed reaction are employed and their effect on acquired resistance evaluated.

### Onset of Delayed-type Hypersensitivity

Mice received $18. \times 10^4$ *Listeria* intravenously. At daily intervals spleen counts and footpad tests were carried out on groups of these mice, with the results shown in Table 21 and Figure 27 (cf. Mackaness and Ackerman, 1962).

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TABLE 21

Onset of Delayed-type Hypersensitivity and Growth of L. monocytogenes in the Spleen

| Day of estimation | Mean Spleen Count (organisms/ml. homogenate) | Mean % Increase in Footpad Thickness at 24 hours * |
|---|---|---|
| 1 | $1.2 \times 10^5$ | 2.8 |
| 2 | $9.5 \times 10^5$ | 2.4 |
| 3 | $5.3 \times 10^6$ | 2.2 |
| 4 | $9.1 \times 10^5$ | 8.4 |
| 5 | $1.9 \times 10^5$ | 21.3 |
| 10 | $2.3 \times 10^2$ | 35.1 |
| 19 | | 24.1 |

* The 24-hour reaction is recorded against the day the test injection was carried out.

TABLE 22

Waning of Delayed-type Hypersensitivity

| Time since infection (weeks) | Mean % Increase in Footpad Thickness at: | |
|---|---|---|
| | 3 Hours | 24 Hours |
| 3 | 10.8 | 39.7 |
| 6 | 15.9 | 33.1 |
| 11 | 14.0 | 21.3 |
| 18 | 10.0 | 9.7 |

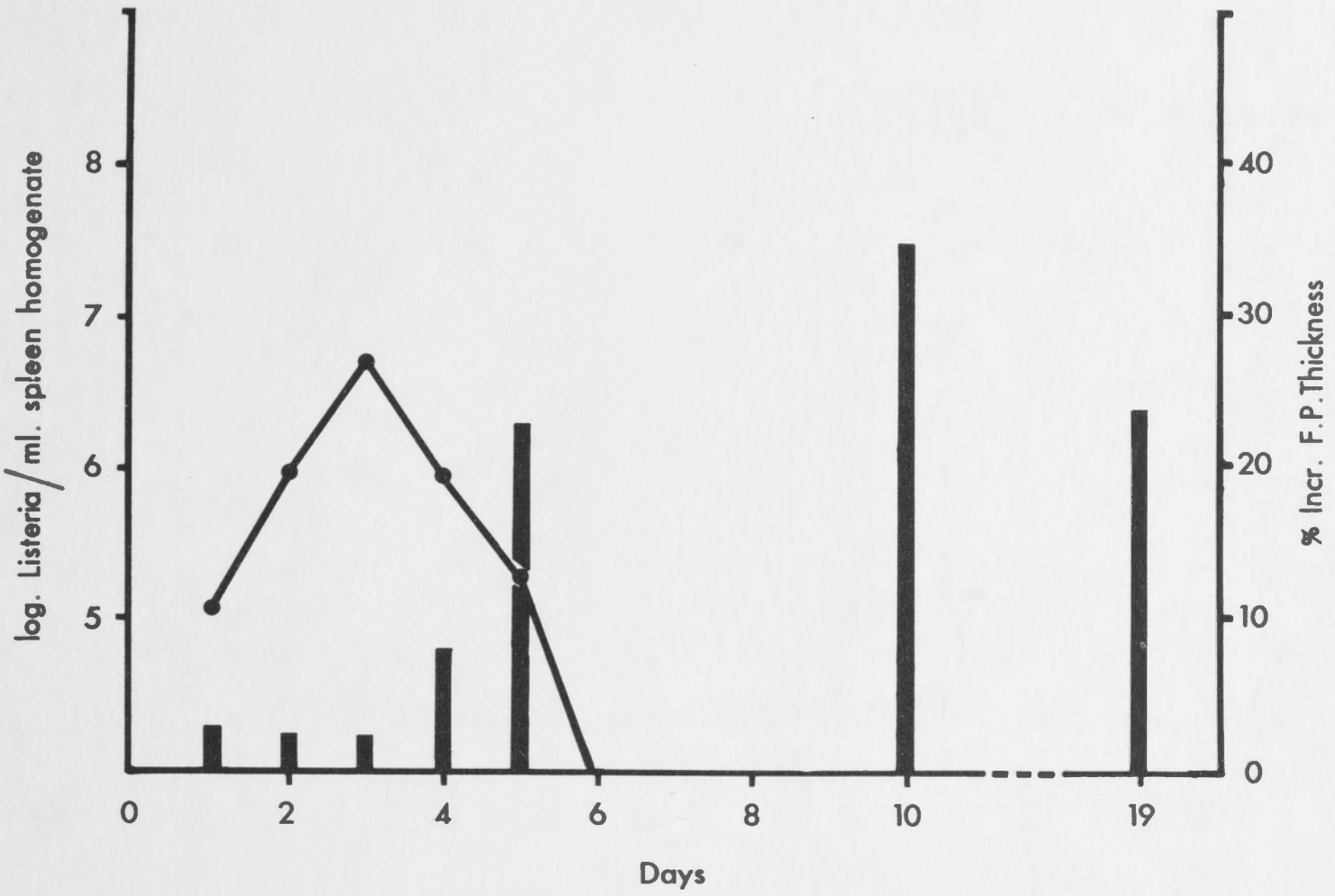


Figure 27.

Serial footpad measurements and spleen counts in mice infected intravenously with $1.8 \times 10^4$ listeriae on Day 0.

It is clear that delayed-type hypersensitivity first becomes detectable at a time when the bacterial population of the spleen is beginning to decline (the differences in the mean footpad reaction on days 3, 4, 5, 8, 10 and 19 are all significant at the 2% level or above). As will be shown below, relatively small numbers of live organisms suffice to inhibit footpad reactivity, so that delayed-type hypersensitivity could probably not be demonstrated earlier by this technique.

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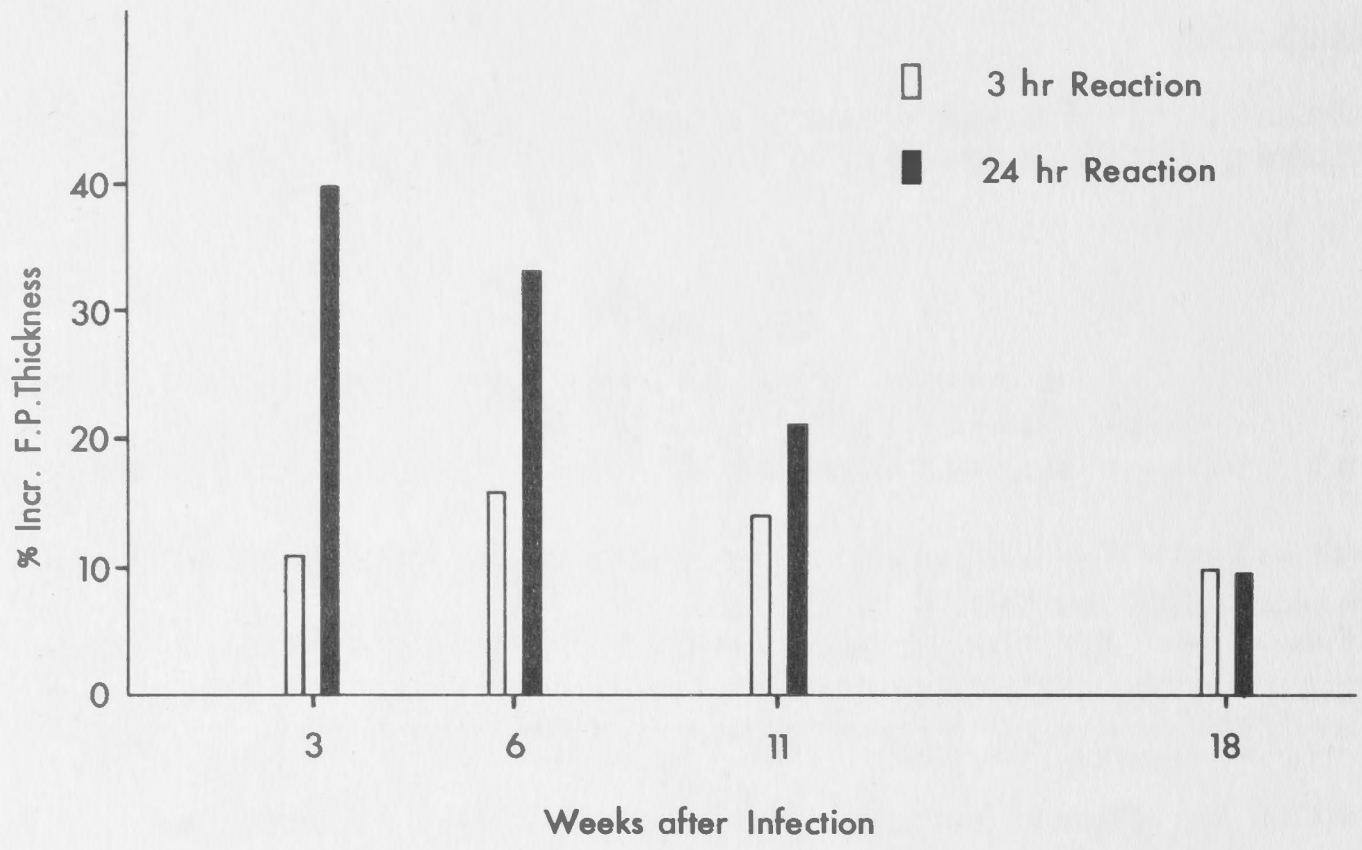


Figure 28.

Mean 3-hour and 24-hour footpad reactions in mice at intervals after intravenous infection with 9 x $10^3$ listeriae.

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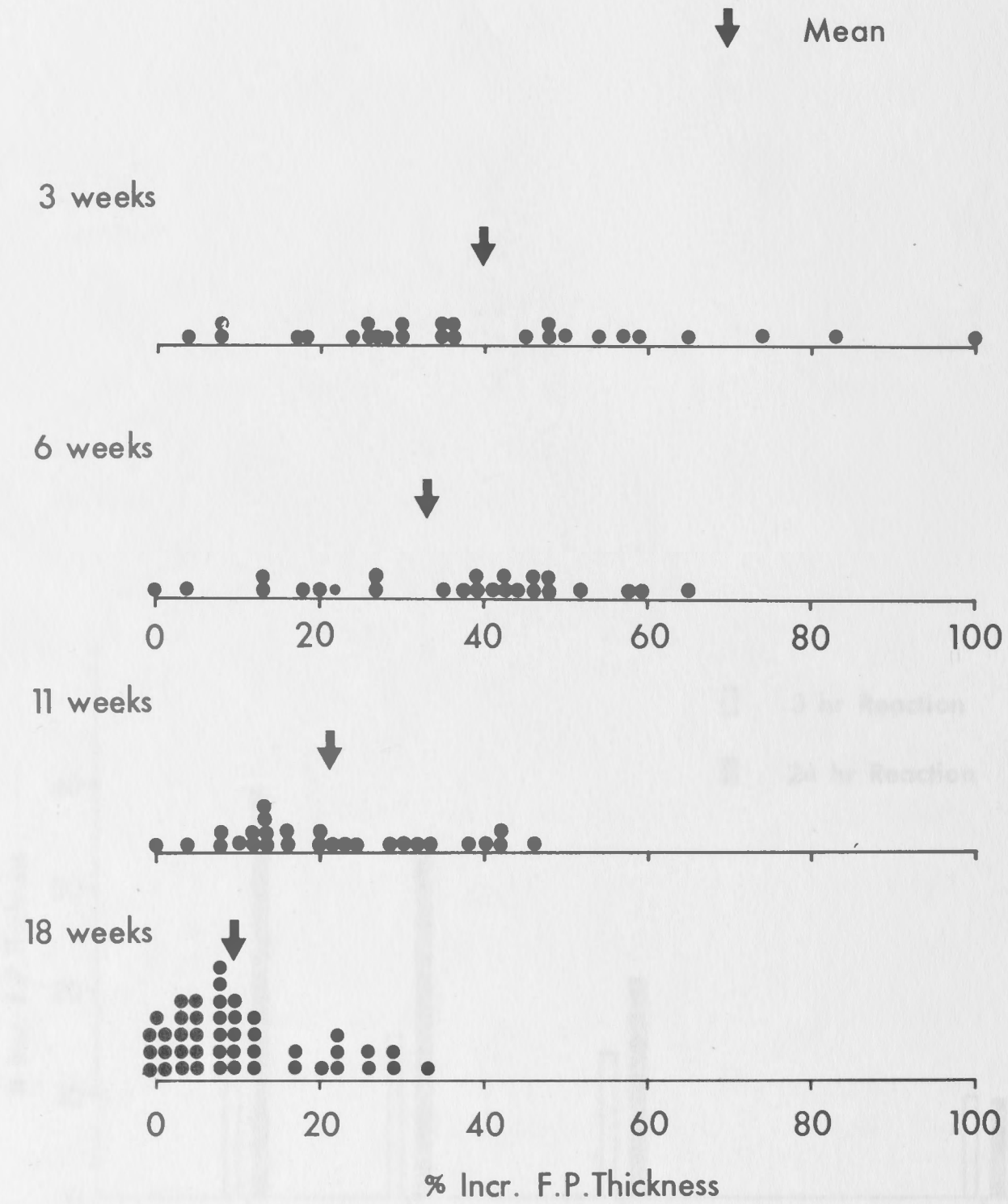


Figure 29.

Individual 24-hour footpad reactions of mice at intervals after intravenous infection with $9 \times 10^3$ listeriae.

Duration of Delayed-type Hypersensitivity. Mice were infected with $9 \times 10^3$ L. monocytogenes by the intravenous route. At intervals footpad tests were carried out. The results appear in Table 22 and Figures 28 and 29.

The highest level of sensitivity was found 3 weeks after infection; the data reported above on the onset of this reaction suggest that reactivity may have been more marked earlier. From this point there was a gradual decline. While the difference between the mean 24-hour reactions at 3 and 6 weeks is not statistically significant, this tendency is found in other experiments and the difference is probably real.

A number of the features of this decline in delayed-type hypersensitivity are apparent on examination of Figure 29. Skin reactions are commonly reported as positive or negative. Here (as often elsewhere)such a procedure would certainly be arbitrary. At no stage do the values fall into two clearly separable groups. Both the mean and the range of the observations decrease steadily. Nevertheless, even 18 weeks after infection, a few individuals show a degree of delayed sensitivity comparable with the average level 15 weeks before.

Duration of Acquired Resistance. Acquired resistance had already been shown to decline in a fashion similar to that of delayed-type hypersensitivity (Mackaness and Ackerman, 1962). It was now decided to investigate this process again, but in a somewhat different manner. What was needed was a measure

TABLE 23

Effect of a Footpad Test on a subsequent Spleen Count

| Experiment | Number of organisms per ml. spleen homogenate | | | | | |
|---|---|---|---|---|---|---|
| | Footpad test performed 24 hours before challenge | | | No Footpad test performed | | |
| | Number in group | Mean | Range | Number in group | Mean | Range |
| I* | 26 | $9.7 \times 10^3$ | $30-5.8 \times 10^4$ | 14 | $5.4 \times 10^3$ | $10^2-3.3 \times 10^4$ |
| II* | 14 | $5.3 \times 10^4$ | $10^2-2.2 \times 10^5$ | 11 | $2.4 \times 10^4$ | $10^2-10^5$ |

* The mice in Experiment I were infected intravenously with $9 \times 10^3$ listeriae 3 weeks before challenge. The challenge consisted of $1.8 \times 10^4$ organisms; spleen counts were carried out 42 hours later (Figure 13 "3 weeks"). The difference in mean spleen count is not significant ($0.4 > P > 0.3$).

** The mice in Experiment II were infected intravenously with $8 \times 10^2$ listeriae 14 weeks before challenge with $2.4 \times 10^4$ listeriae. Spleen counts were performed 68 hours later (Figure 14). The difference in mean spleen count is not significant ($0.7 > P > 0.6$).

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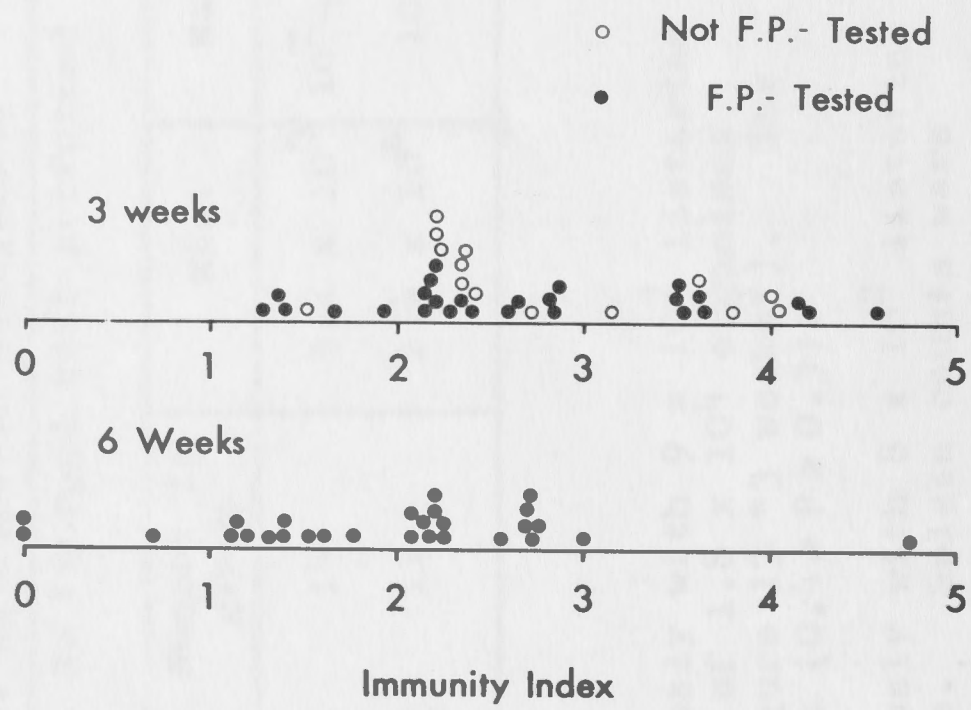


Figure 30.

Immunity indices of mice 3 and 6 weeks respectively after infection with 9 x $10^3$ listeriae. Spleen counts were performed 42 hours after challenge. The values indicated by circles were obtained in mice which had not undergone a footpad test 24 hours before challenge.

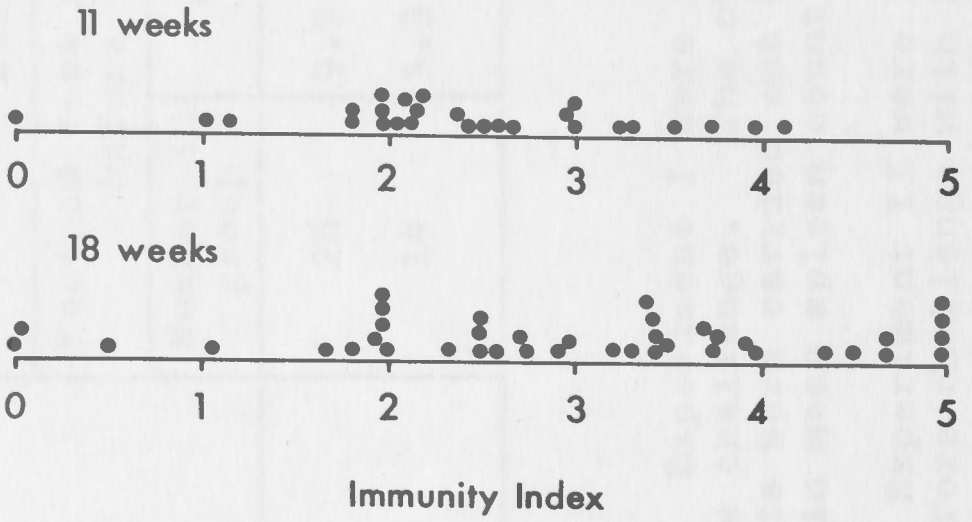


Figure 31.

Immunity indices of mice 11 and 18 weeks respectively after infection with 9 x $10^3$ listeriae. Spleen counts were performed 68 hours after challenge.

of the acquired resistance of an individual at a given point in time, for comparison with that individual's delayed reaction. For this purpose the "Immunity Index" was used (see Chapter I). As this necessitated carrying out a footpad test followed by challenge and spleen count on each mouse, the effect of such an injection of listerin on the subsequent spleen count had to be established.

On two occasions a challenge was carried out on groups of mice previously infected under the same conditions and some of which had received an injection of listerin into the foot 24 hours before. In Table 23 are recorded the spleen counts of these groups, while Figures 30 and 32 show the individual "Immunity Indices" calculated from the spleen count and the mean spleen count of the normal controls. In neither experiment was there evidence that the footpad test had affected the spleen count in any way.

It was thus permissible to assess both delayed-type hypersensitivity and acquired resistance in the same animals. Mice used to investigate the duration of delayed-type hypersensitivity (see Table 22) were challenged immediately after the footpad test had been read, along with uninfected controls. From what was already known of the decay of acquired resistance it was thought that no evidence of resistance would be found, in some mice at least, before the third day, if the original infection had taken place much more than 9 weeks before (see Figure 6, Mackaness and Ackerman, 1962). Spleen counts were therefore carried out after an

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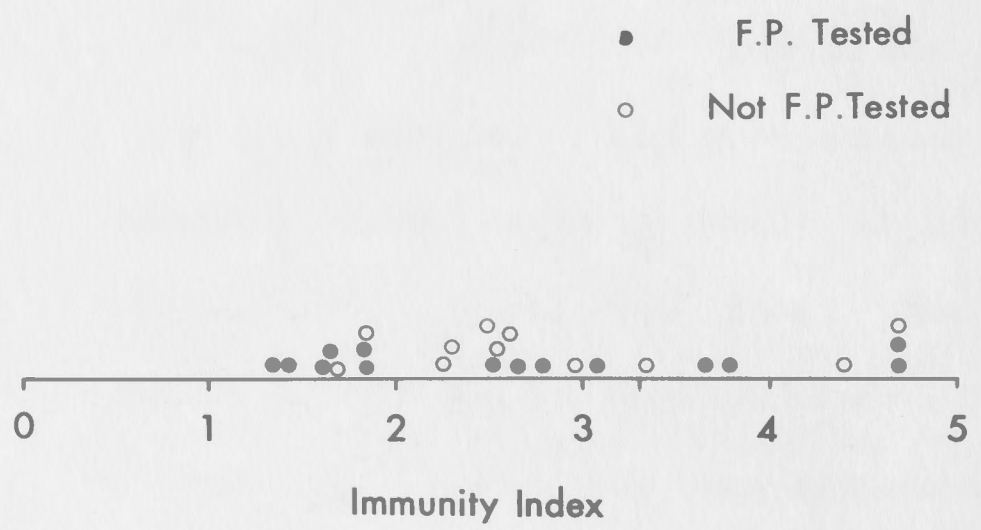


Figure 32.

Immunity indices of mice 14 weeks after intravenous infection with 8 x $10^2$ listeriae. Spleen counts were performed 68 hours after challenge. The values indicated by circles were obtained in mice which had not undergone a footpad test 24 hours before challenge.

TABLE 24

Waning of Acquired Resistance

| Time since infection (weeks) | Mean Spleen Count (organisms/ml. homogenate) | |
|---|---|---|
| | Convalescent Mice | Normal Mice |
| 3* | $9.7 \times 10^3$ | $1.1 \times 10^6$ |
| 6* | $7.3 \times 10^4$ | $2.8 \times 10^6$ |
| 11** | $7.4 \times 10^4$ | $6.3 \times 10^6$ |
| 18** | $6.3 \times 10^5$ | $9.3 \times 10^6$ |

* Spleen counts were carried out 42 hours after challenge.

** Spleen counts were carried out 68 hours after challenge.

interval of 2 or 3 days. The data thus obtained appear in Table 24 and Figures 30 and 31.

Table 24 shows acquired resistance to decline in much the same fashion as was indicated by the earlier work. Figures 30 and 31, in which are given the "immunity indices" of all mice, bring out the great variability in the level of resistance at all stages. While these results do not permit of direct comparison between animals challenged at 3 weeks and 18 weeks after infection, as spleen counts were not carried out at the same interval after challenge, it is obvious that even after this latter period some mice display a resistance not greatly inferior to that of their fellows 15 weeks earlier.

Comparison of Table 22 with Table 24 and of Figures 29 with Figures 30 and 31 show that both delayed-type

TABLE 25

Levels of Delayed-type Hypersensitivity and Acquired Resistance after a primary and challenge infection

| Nature of infection | Mean % Increase in Footpad Thickness at: | | Individual Spleen Counts (organisms/ml. homogenate) |
|---|---|---|---|
| | 3 Hours | 24 Hours | |
| Primary | 19.6 | 30.3 | $1.5 \times 10^3$, 220, 900, 860, 5, 5, 5 and < 5* |
| Challenge | 20.0 | 30.5 | 90, 30, 10, < 5, < 5, < 5, < 5. |

* Spleen homogenates were so diluted that bacterial populations of less than 5 organisms/ml. were likely to remain undetected.

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hypersensitivity and acquired resistance decline in similar fashion, though the former reactivity seems somewhat more evanescent than the latter.

In order to decide if the parallelism between these two immunological states was a general feature of listeric infection, the levels of both were assessed in several experiments where variation in the conditions of infection might favour one response at the expense of the other.

Effect of Re-infection. Mice that have overcome a primary infection with ca $10^4$ listeriae possess a high degree of delayed reactivity and their degree of acquired resistance now enables them to survive much heavier infections. The effects of a second infection were now investigated. Mice received intravenous injections of the following materials on the days indicated:

| | Day 0 | Day 37 |
|---|---|---|
| "Primary Infection" | B.S.S. | $10^4$ listeriae |
| "Challenge Infection" | $10^4$ listeriae | $10^6$ listeriae |

Four weeks later footpad tests were carried out on animals from each group, while others were challenged for spleen count 48 hours afterwards. The results appear in Table 25.

Unfortunately the challenge was small ($4 \times 10^3$ organisms); this probably accounts for the number of apparently sterile spleens. Statistically no significant difference can be shown between the two groups, owing to the large variance of the "Primary Infection" group, but inspection of Table 25 certainly suggests that the "Challenge Infection" group displayed a uniform and marked bactericidal ability which is not evident in all members of the other group. With respect

to footpad reactivity the two groups are identical.

Effects of Variation in Dose and Route of Injection. In the three experiments to be described, the levels of delayed sensitivity and resistance produced by infection with large and small inocula by the intravenous, intraperitoneal and subcutaneous routes are compared; an infection by $10^4$ listeriae intravenously was used as the "reference standard" in each case.

Mice received intravenous injections of $10^5$, $10^4$, and $10^2$ viable units of L. monocytogenes. Three weeks later footpad tests and spleen counts were carried out. Table 26 records the data obtained.

TABLE 26

Levels of Delayed-type Hypersensitivity and Acquired Resistance after intravenous infections

| Inoculum given | Mean % Increase in Footpad Thickness at: | | Mean Spleen Count (organisms/ml. homogenate) |
|---|---|---|---|
| | 3 Hours | 24 Hours | |
| $10^2$ | 13.4 | 19.3 | $2.4 \times 10^4$ |
| $10^4$ | 14.3 | 30.3 | $3.7 \times 10^2$ |
| $10^5$ | 14.6 | 24.3 | $1.6 \times 10^2$ |

The level of delayed sensitivity in the "$10^2$" group is significantly lower than that of the "$10^4$" group, while the "$10^5$" group is not statistically distinct from either of the

others in this respect. On two other occasions the footpad reactions of animals infected with $10^5$ and $10^4$ listeriae have been found to be virtually identical; in one such experiment the mean increase in footpad thickness was 28.4% and 24.8% respectively, whereas inocula of $10^2$ organisms always yield a considerably lower level of delayed sensitivity than does infection with $10^4$ bacteria. In acquired resistance also the "$10^2$" group is notably inferior ($P < 0.001$), while the other two groups are indistinguishable.

To determine the effects of subcutaneous infection, inocula of $10^4$ and $10^6$ organisms were administered subcutaneously and other mice were given $10^4$ organisms intravenously. Table 27 indicates the levels of footpad reactivity and splenic antibacterial powers displayed by them three weeks later.

TABLE 27

Levels of Delayed-type Hypersensitivity and Acquired Resistance after subcutaneous infections

| Inoculum and route of inoculation | Mean % Increase in Footpad Thickness at: | | Mean Spleen Count (organisms/ml. homogenate) |
|---|---|---|---|
| | 3 Hours | 24 Hours | |
| $10^4$: Subcutaneous | 19.7 | 16.6 | $4 \times 10^4$ |
| $10^6$: " | 18.0 | 18.6 | $2.5 \times 10^4$ |
| $10^4$: Intravenous | 21.3 | 27.1 | $2.0 \times 10^3$ |

The delayed reactivity of the intravenously infected group is significantly higher than that of the other two

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TABLE 28

Levels of Delayed-type Hypersensitivity and Acquired Resistance after Intraperitoneal infections

| Inoculum and route of inoculation | Mean % Increase in Footpad Thickness at: | | Mean Spleen Count (organisms/ml. homogenate) |
|---|---|---|---|
| | 3 Hours | 24 Hours | |
| $10^4$: Intra-peritoneal | 19.6 | 18.0 | $3.6 \times 10^5$ |
| $10^6$: Intra-peritoneal | 24.3 | 25.0 | $10^5$ |
| $10^4$: Intra-venous | 22.1 | 33.9 | $4.9 \times 10^3$ |

TABLE 29

Statistical Significance of Differences in Footpad Reactivity and Mean Spleen Count indicated in the data of Table 28

| Groups compared | Probability that the observed differences in magnitude of the response indicated are due to chance - | |
|---|---|---|
| | Delayed Reaction | Mean Spleen Count |
| $10^4$ I.V. & $10^6$ I.P. | $< 0.05$ | $< 0.005$ |
| $10^6$ I.P. & $10^4$ I.P. | $< 0.05$ | $< 0.01$ |

($P < 0.001$ and $< 0.01$ respectively) and the same is true of the mean spleen count ($P < 0.001$ and $< 0.02$ respectively), whereas the two subcutaneously infected groups are not distinguishable with respect to either parameter.

A similar experiment was performed to evaluate the effects of intraperitoneal infection. The results are reported in Table 28; Table 29 indicates their statistical significance.

It is seen that the three groups can be arranged in the same order of increasing reactivity with respect to both delayed-type hypersensitivity and acquired resistance, i.e. the parallelism between the two responses is complete. Other mice from these groups were used to estimate the $LD_{50}$ (see Table 2, Chapter II); the estimates of the relative levels of acquired resistance provided by the two methods were not consistent, one with the other, and the possible reasons for these discrepancies were discussed in Chapter II.

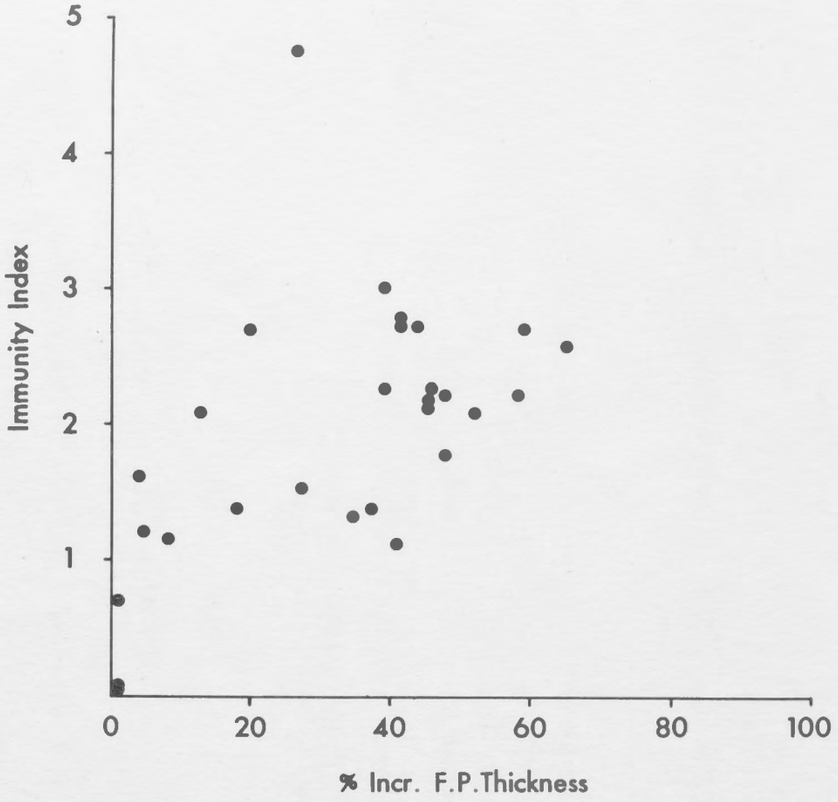


Figure 33.

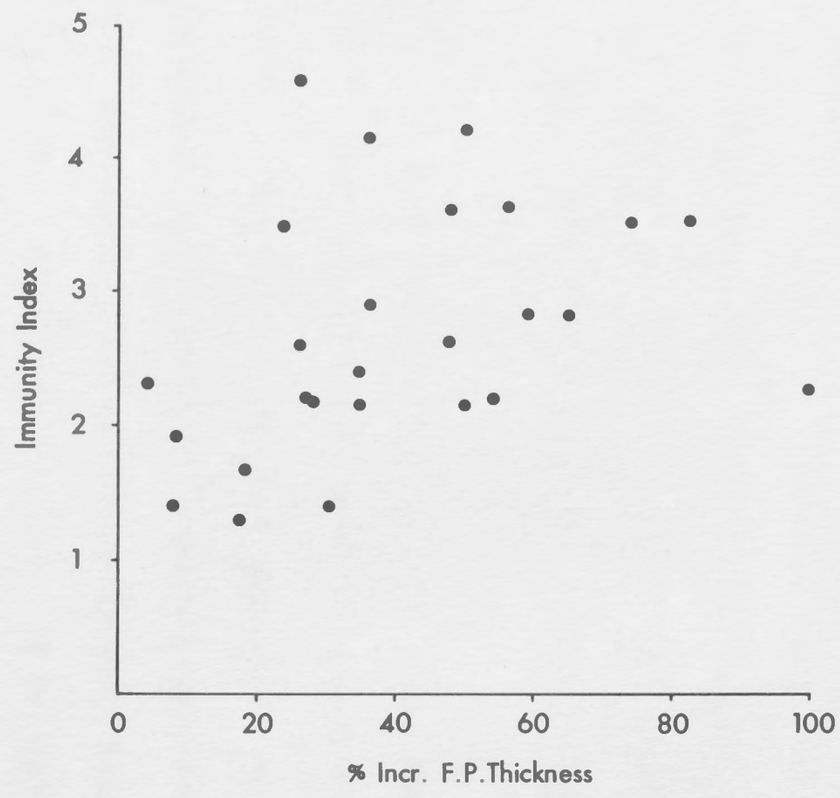


Figure 34.

Relation of Delayed-type Hypersensitivity to Acquired Resistance in individual mice 3 weeks (Figure 33) and 6 weeks (Figure 34) after intravenous infection with 9 x $10^3$ listeriae.

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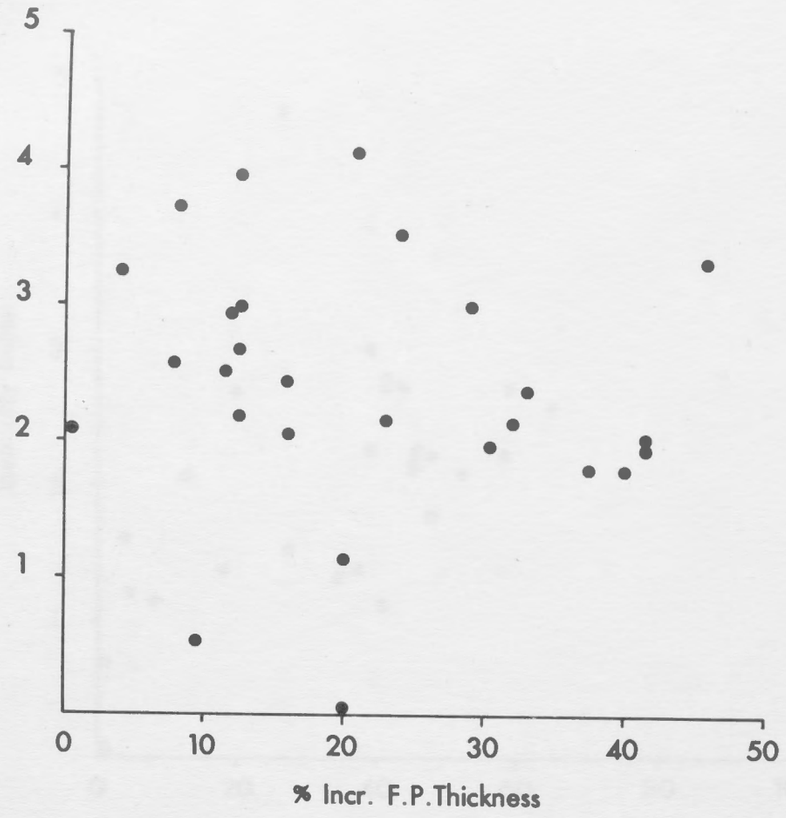


Figure 35.

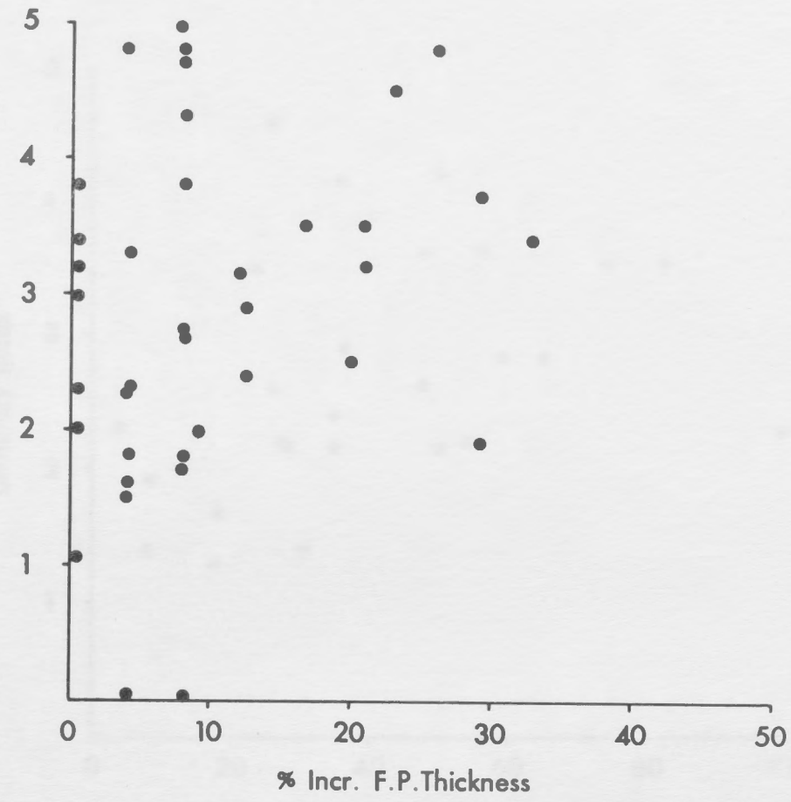


Figure 36.

Relation of Delayed-Type Hypersensitivity to Acquired Resistance in individual mice 11 weeks (Figure 35) and 18 weeks (Figure 36) after intravenous infection with $9 \times 10^3$ listeriae.

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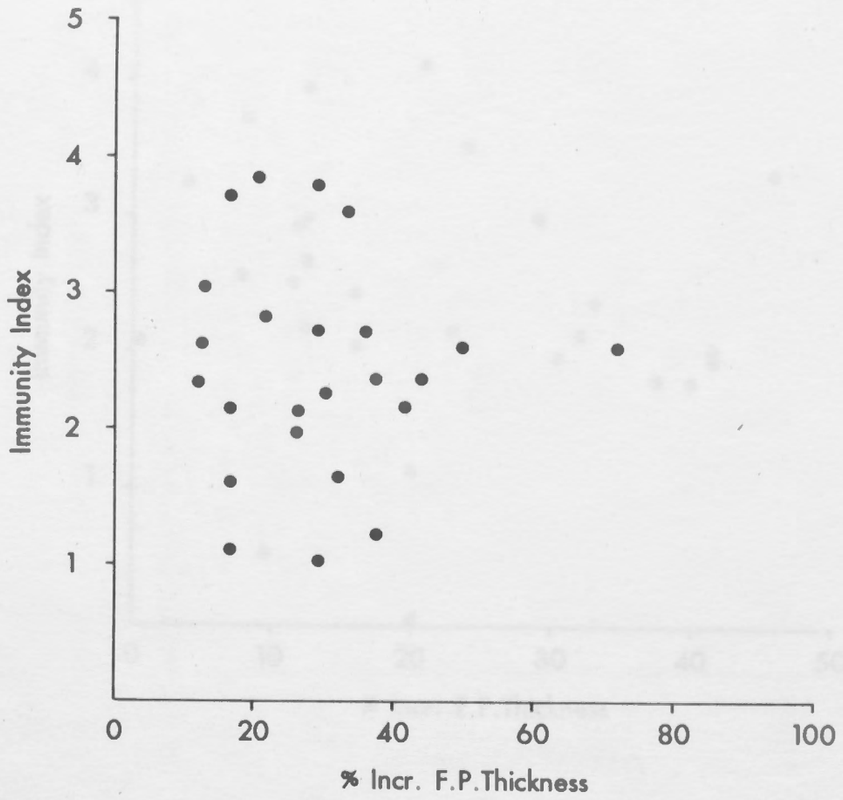


Figure 37.

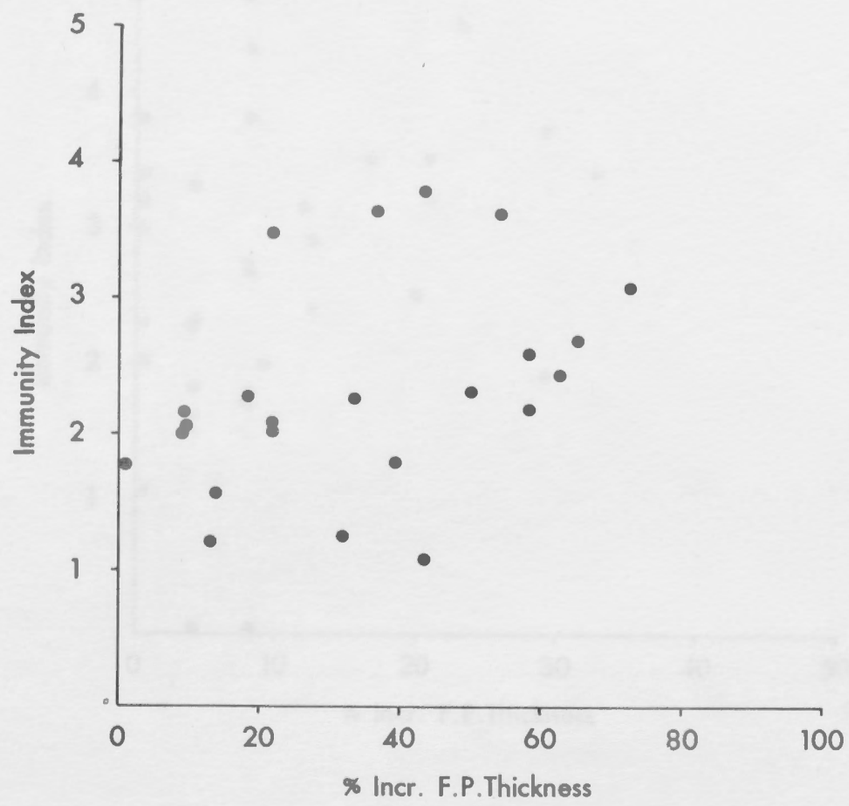


Figure 38.

Relation of Delayed-Type Hypersensitivity to Acquired Resistance in individual mice 4 weeks after intravenous infection with $10^4$ listeriae (Figure 37) and with $10^2$ listeriae (Figure 38).

# CHAPTER VI.

## DELAYED-TYPE HYPERSENSITIVITY AND ACQUIRED RESISTANCE

### B. The Relationship in Individuals

The results of the experiments detailed in Chapter V may be broadly summarized in the statement that the degree of delayed sensitivity in general paralleled that of acquired resistance. The expression of these two reactivities was considered at the level of the group only. It would obviously be desirable to compare both responses in the same animal. As it had been shown that the spleen count was unaffected by a footpad test 24 hours before challenge (see p.116), individual values of these two parameters were derived from a number of experiments and examined further.

The procedure adopted was as follows. For each mouse there was available a record of the delayed reaction and of the spleen count after challenge. The "immunity index" was calculated and the footpad reaction plotted against it, to yield scatter diagrams such as are shown in Figures 33 to 38. In a number of experiments the correlation coefficient was also calculated (see Chapter I). For this calculation the footpad reaction was represented by the log (% increase in footpad thickness)$^2$, partly for convenience and also because there was a suggestion from other scatter diagrams that the correlation between the square of the footpad reaction and the

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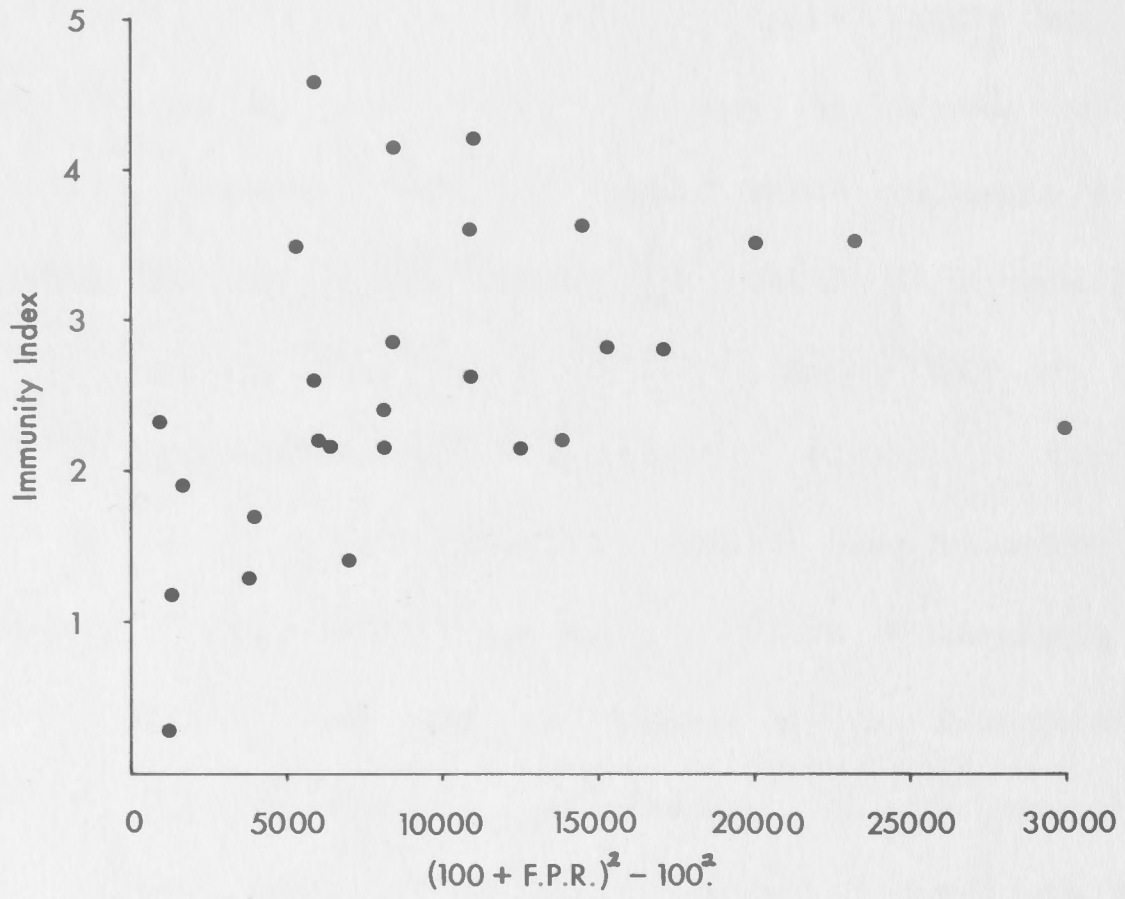


Figure 39.

Relation of immunity index to increase in cross-sectional area of footpad represented by $[(100 + FPR)^2 - 100^2]$. The same data are used as appear in Figure 33.

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## TABLE 30

### Details of the Experiments from which the Data of Figures 16-22 were derived

| Experiment yielding the data of: | Inoculum* | Time elapsed between infection and evaluation of immune response | Correlation** Coefficient |
|---|---|---|---|
| Figure No. | | | |
| 16 & 22 | $9 \times 10^3$ | 3 weeks | + 0.453*** |
| 17 | " | 6 " | + 0.285 |
| 18 | " | 11 " | - 0.270 |
| 19 | " | 18 " | + 0.240 |
| 20 | $10^4$ | 4 " | |
| 21 | $10^2$ | 4 " | |

* All groups were infected by the intravenous route.

** The Correlation Coefficient (r) is calculated from the individual values for % increase in footpad thickness and immunity index.

*** In this case alone r is significant, at the 5% level.

"immunity index" might be closer. Some details of the experiments on which Figures 33 to 38 are based are given in Table 30, together with the correlation coefficients.

It can be argued that a better assessment of the intensity of delayed reactions is achieved if the increases in two dimensions are considered. This is the method used by Dietrich, Nordin and Bloch (1962). It was thought that a similar end might be achieved by calculating from each footpad reading a quantity which would, to some extent, reflect the increase in cross-sectional area of the foot. As the increase in footpad thickness is expressed as a percentage, the thickness of the foot before injection is represented by 100, its thickness after injection by (100 + the Foot-Pad Reaction as a percentage) and the cross-sectional area after injection is then represented by $(100 + FPR)^2$. The increase in cross-sectional area is then $[(100 + FPR)^2 - 100^2]$. The footpad reactions indicated in Figure 33 were thus treated and then plotted against the "immunity index" as before, to yield Figure 39, It is not difficult to see that none of these manipulations revealed any important correlation between the levels of delayed-type hypersensitivity and acquired resistance in individual mice.

In one case, the experiment represented in Figures 33 and 39, the value of r, the correlation coefficient, obtained was significant ($P < 0.05$). The importance of this result is not clear, since such a correlation did not appear in the other results of the same experiment. (All the animals in

the experiments of Figures 33-36 were injected at the same time with the same bacterial suspension). In any event, a value for r of 0.453 indicates that only 21% of the variation in the parameters treated is correlated.

## Relationship between Delayed-type Hypersensitivity and Survival

Another possibility was that the level of delayed reactivity might afford some indication of the animal's response to challenge as assessed by its subsequent death or survival. Mice received an intravenous dose of $8 \times 10^2$ listeriae; a small inoculum was given in order that the subsequent levels of delayed reactivity and resistance might cover a wide range (cf. Figure 38). On day 17 they were marked for identification and footpad tests were carried out. On day 97 (14 weeks after the original infection) they received a challenge dose of $10^6$ viable units of L. monocytogenes. Deaths were recorded over the following two weeks. In Table 31 the results of this challenge are shown.

The convalescent mice still possessed some degree of immunity, as their survival differs significantly from that of the controls (chi-square = 15.54, $P < 0.005$).

TABLE 31

Relationship of Footpad Reaction 17 days after infection to survival 97 days after infection

| Fate | Normal Mice | Convalescent Mice | Mean Footpad Reaction of Convalescent Mice |
|---|---|---|---|
| Survived | 6 | 91 | 15.5 |
| Died | 18 | 43 | 19.9 |

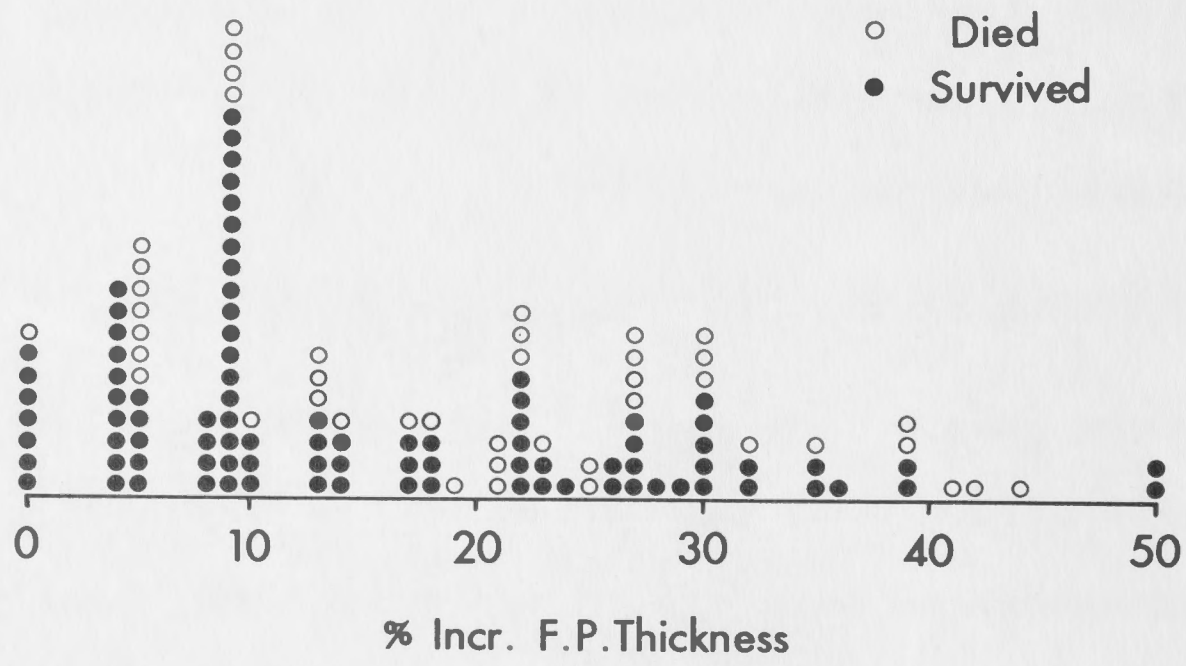


Figure 40.

Relationship of individual 24-hour footpad reaction to survival. Footpad tests were carried out 17 days after infection with 8 x $10^2$ viable units of L. monocytogenes. Mice were challenged on Day 97.

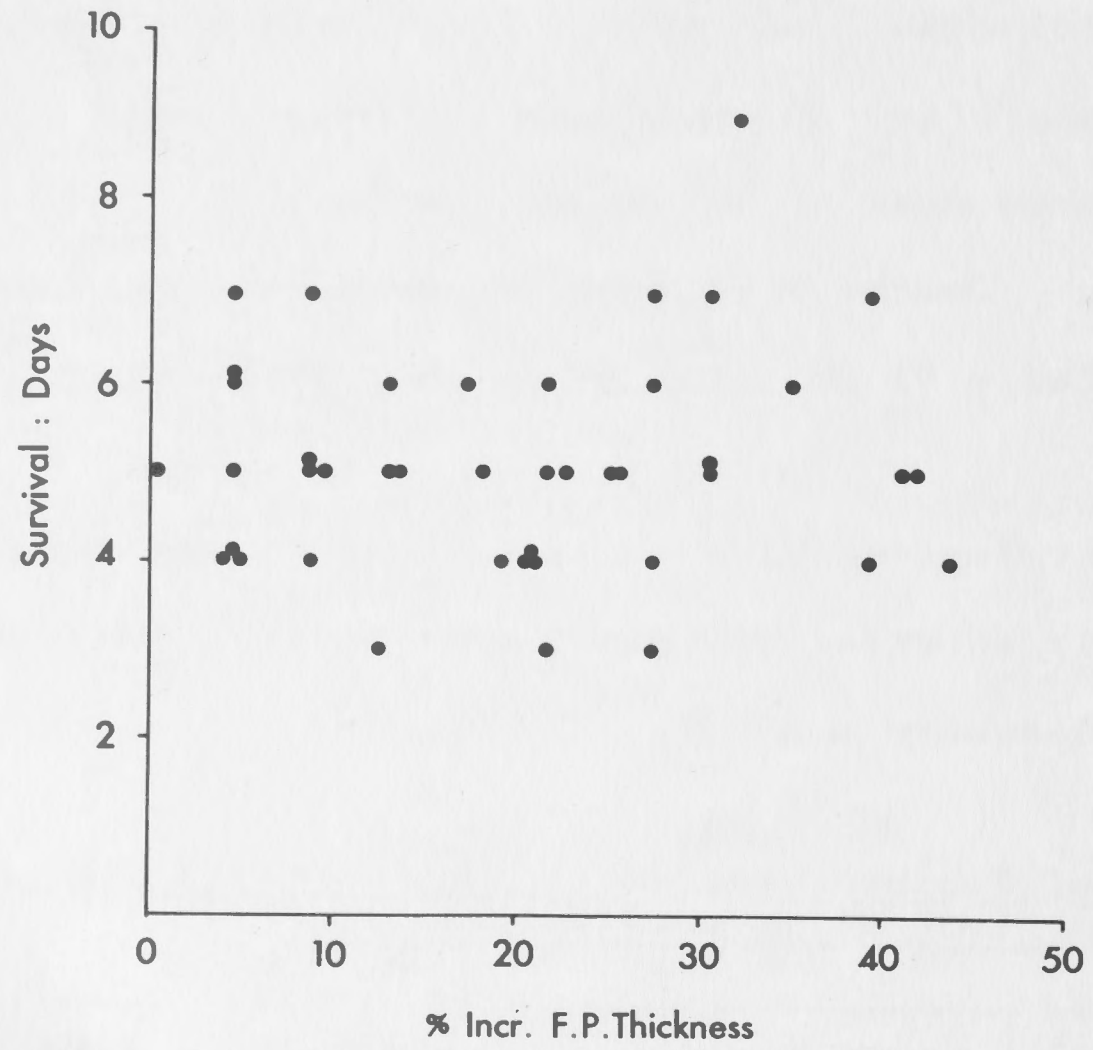


Figure 41.

Relationship of individual 24-hour footpad reaction to duration of survival after challenge (the same experiment as is described in Figure 40).

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TABLE 32

Mortality-rates in Groups of Mice classified with respect to size of Footpad Reaction

| Range of Footpad reaction (% Increase F-p Thickness) | Number of Mice which: | |
|---|---|---|
| | Survived | Died |
| 0 - 10 | 47 | 13 |
| 10 - 20 | 13 | 7 |
| 20 - 30 | 17 | 13 |
| 30 + | 14 | 10 |

TABLE 33

Mortality-rates in Mice treated with "B.S.S.-Adjuvant" and "Listerin-Adjuvant" infected with Listeria and later challenged

| Treatment given before primary infection | % Survival after challenge | Mean 24-hour Reaction* in | |
|---|---|---|---|
| | | Survivors | Dead |
| B.S.S. in incomplete adjuvant | 32 | 25.9 | 28.9 |
| *Listeria* antigens in incomplete adjuvant | 53 | 45.3 | 39.6 |

* Measured 24 days before challenge.

The mean footpad reaction of those mice destined to survive is significantly lower than the reaction in those which were to die, although its statistical importance is not great ($0.05 > P > 0.025$).

In Figure 40 the individual footpad reactions of all mice are indicated, together with their subsequent fate. No clear-cut relationship is obvious. In Table 32 these mice are classified according to size of footpad reaction and response to challenge. The distribution of deaths throughout the groups is without significance when compared with the overall mortality (chi-square = 5.80, $0.6 > P > 0.5$). Similarly the difference in mortality between the "negative" reactors (% increase in footpad thickness 0-10%) and the "positive" reactors (% increase in footpad thickness greater than 10%) is not meaningful ($0.2 > P > 0.1$).

In Figure 41 the survival time in days is plotted against the footpad reaction. One sees, for example, that the reactions of animals dying on the fourth day had ranged between 4 and 44%, and that animals with a reaction around 30% survived 3 to 9 days. In neither case, therefore, is a correlation between delayed sensitivity and survival revealed.

In Chapter IV it was shown that injection of mice with preparations of Listeria antigens in incomplete adjuvant before infection resulted in very marked 24-hour reactions and evidence was presented to show that these reactions were a manifestation of delayed-type hypersensitivity. The response of these mice to a subsequent infection was examined. They

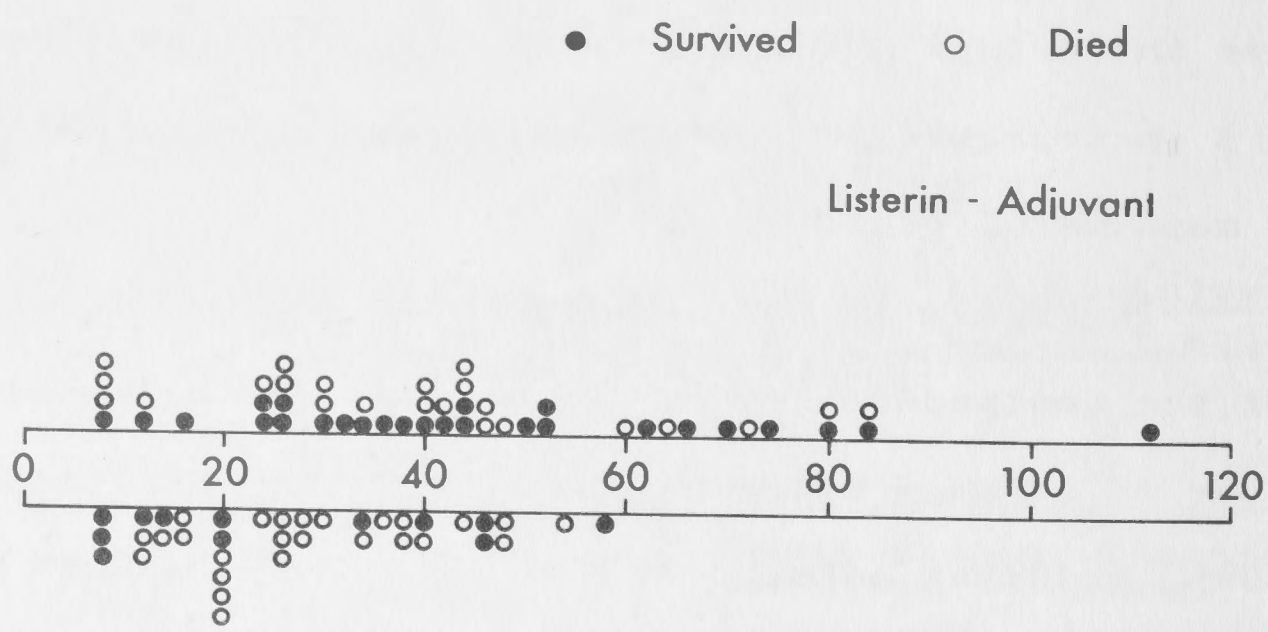


Figure 42.

Mice were injected with Listeria antigens in incomplete adjuvant before infection with L. monocytogenes. Controls received B.S.S. in adjuvant. Footpad tests were performed 24 days after primary infection and the mice were challenged 44 days after primary infection. The figure shows the relation of the individual footpad reaction to survival (the central scale indicates the percentage increase in footpad thickness).

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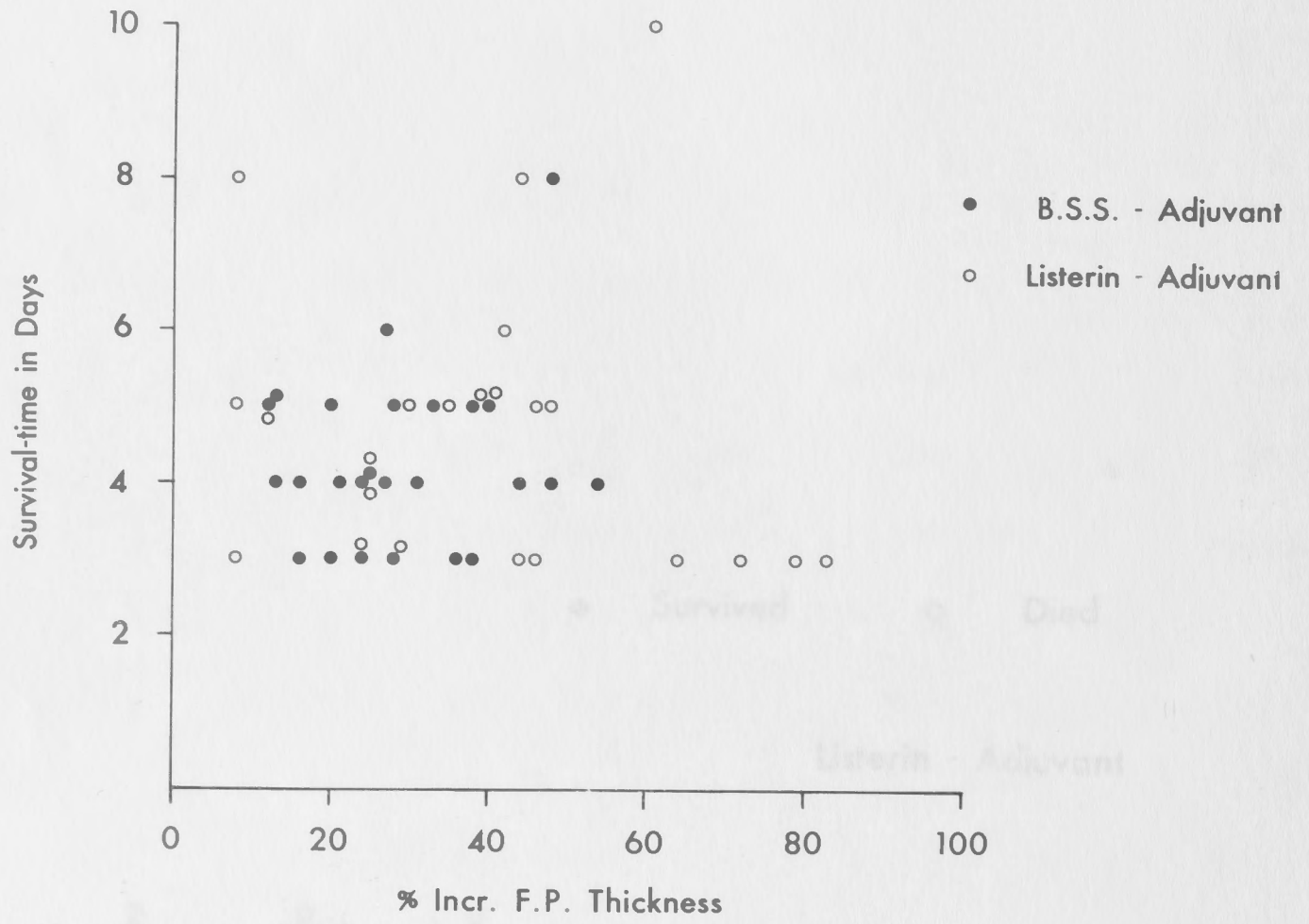


Figure 43.

Mice were injected with Listeria antigens in incomplete adjuvant before infection with L. monocytogenes. Controls received B.S.S. in adjuvant. Footpad tests were performed 24 days after primary infection and the mice were challenged 44 days after primary infection. The figure shows the relation of the individual footpad reaction to duration of survival.

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were challenged with $3.2 \times 10^7$ L. monocytogenes by the intravenous route 4 days after their initial infection. Some data derived from the results of this experiment are presented in Table 33.

The difference in survival rate between those mice injected with Listeria antigens and with B.S.S. is not significant, nor are the survivors within each treatment group distinguished from those which died by a difference in mean footpad reactivity. In Figures 42 and 43 the relationships between delayed reaction and survival or death and between delayed reaction and length of survival are indicated. The same lack of correlation is seen as was apparent in Figures 40 and 41.

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TABLE 34

Effect of pre-treatment with Listeria antigens on survival after primary infection and on development of delayed-type hypersensitivity

| Injections given before infection | % Survival* after infection with $6.6 \times 10^4$ Listeria | Mean % Increase in Footpad Thickness at 24 hours |
|---|---|---|
| Listerin | 46.0 | 19.8 |
| Formalin-killed Listeria | 57.3 | 34.0 |
| B.S.S. | 50.5 | 30.8 |

* Each group contained more than 100 mice.

TABLE 35

Effect of Listeria Vaccines on Resistance to a primary infection

| Vaccine given (1 i.v. dose) | Mean Spleen Count 48 hours after primary infection |
|---|---|
| Formalin-killed Listeria | $6.3 \times 10^7$ |
| Heat-killed Listeria ($70^o$ for 30') | $1.9 \times 10^7$ |
| " " ($100^o$ for 5') | $4.8 \times 10^7$ |
| B.S.S. (negative control) | $6.4 \times 10^7$ |
| $10^4$ live Listeria | $1.5 \times 10^4$ |

# CHAPTER VII.

## DELAYED-TYPE HYPERSENSITIVITY AND ACQUIRED RESISTANCE

### C. Experimental Modification of the Responses

The simplest method of investigating the relationship which concerns us here would be to induce artificially the one immunological response in the absence of the other. This was attempted in various ways.

Effects of Vaccines. Killed vaccines have been shown to have a protective effect only in tuberculosis, not in other infections caused by facultative intracellular bacterial parasites. In the section on the induction of delayed sensitivity (Chapter IV) the result of treating mice with injections of listerin and formalin-killed Listeria was examined. Reference to the experiment reported in Table 16 (p. 101 ) shows that these animals received 5 doses of these materials and were then challenged. The survival rates and the mean 24-hour reactions of the survivors are given in Table 34.

Thus listerin depresses the footpad reaction markedly without affecting survival, and formalin-killed organisms apparently do not modify either aspect of the response to infection.

Table 35 presents the spleen counts of animals which had received a single intravenous dose of the vaccines indicated 2 weeks before challenge. The vaccines conferred

TABLE 36

Delayed-type Hypersensitivity and Acquired Resistance in Mice treated with Listeria antigens in complete adjuvant

| Treatments given and route of injection | Mean* % Increase in Footpad Thickness at: | | Mean Spleen Count 72 hours after challenge with $4 \times 10^3$ *Listeria* |
|---|---|---|---|
| | 3 Hours | 24 Hours | |
| B.S.S. - Adjuvant+ s.c. | 13.4 | 2.5 | $1.6 \times 10^5$ |
| *Listeria* i.v. - Adjuvant s.c. | 19.5 | 26.7 | (not done) |
| *Listeria* i.v. | 21.2 | 34.9 | 20 |
| Formalin-killed *Listeria*ø in adjuvant s.c. | 23.2 | 13.2 | $1.2 \times 10^5$ |
| Listerin - Adjuvant | 37.0 | 31.1 | $8.6 \times 10^3$ |

* The number in each group was 25, except in the listerin-adjuvant group, where only 14 remained, a number having been discarded in error.

\+ Freund's complete adjuvant was used in each case.

ø The suspension contained $10^{10}$ organisms per ml.

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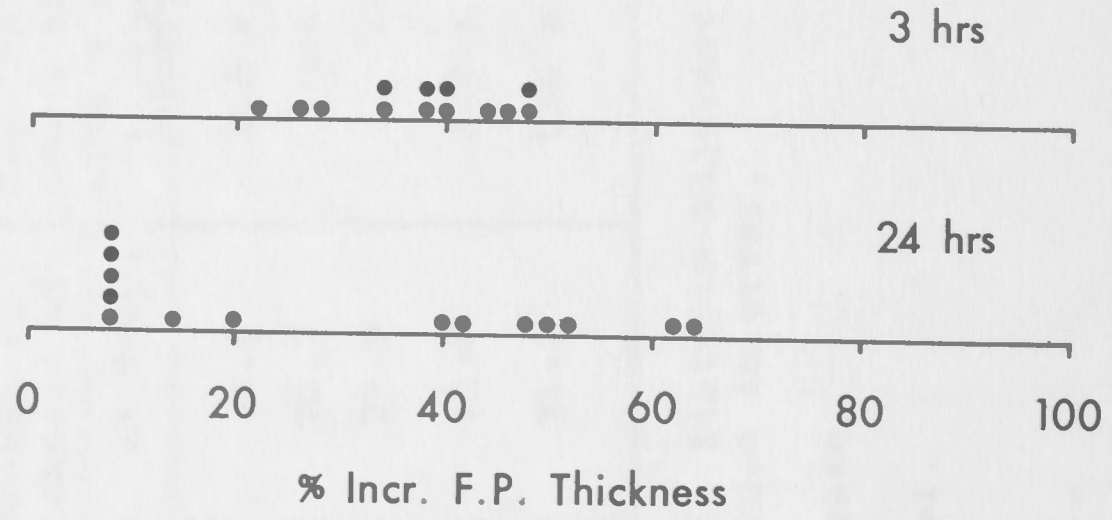


Figure 44.

Individual footpad reactions of mice treated with listerin in complete adjuvant.

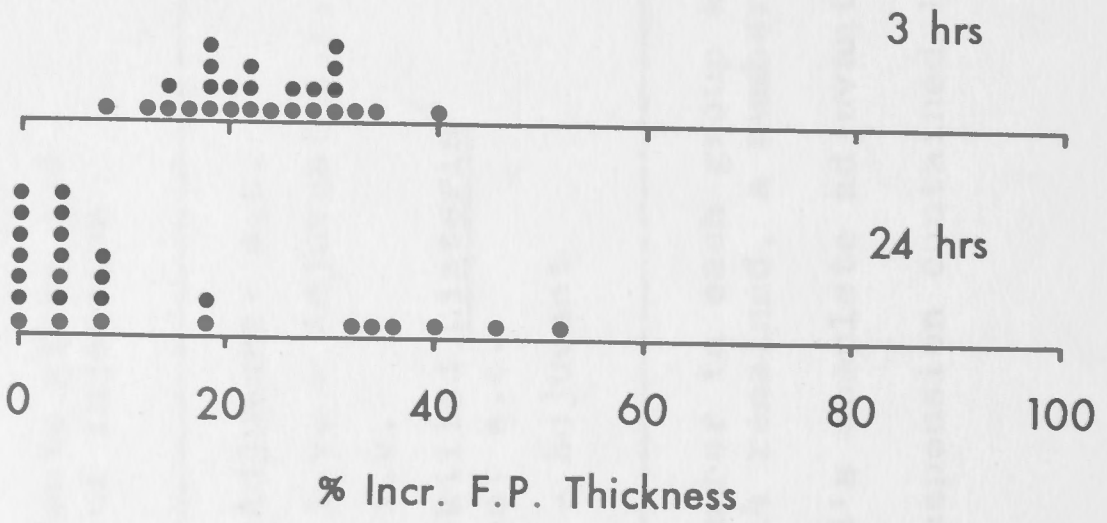


Figure 45.

Individual footpad reactions of mice treated with formalin-killed listeriae in complete adjuvant.

no detectable protection.

By means of this technique or by challenge and by footpad tests it was found that the preparations listed below produced neither acquired resistance nor delayed-type hypersensitivity, whether administered in one or 4-6 doses in saline subcutaneously or intraperitoneally or as a single dose incorporated in incomplete adjuvant:

- Listeria, killed by heat, formalin, diethyl ether, ethanol, phenol, acetone, trichloracetic acid;
- microsomal and protoplasmic fractions of disrupted Listeria;
- listerin.

Effect of Complete Adjuvant

The effect of incorporating the antigen in Freund's complete adjuvant was next examined. Mice received subcutaneous injections of 0.2 ml. of the materials listed in Table 36, mixed with an equal volume of complete adjuvant. Two groups were also inoculated intravenously with $9 \times 10^3$ Listeria. Six weeks later footpad tests and spleen counts were carried out, with the results indicated in Table 36. The individual reactions of the groups injected with formalin-killed Listeria and listerin in adjuvant appear in Figures 44 and 45, where the 24-hour reactions are seen to fall into two fairly distinct classes. Reasons have already been given (Chapter IV) for considering 24-hour reactions of this magnitude in mice to be due to delayed-type hypersensitivity.

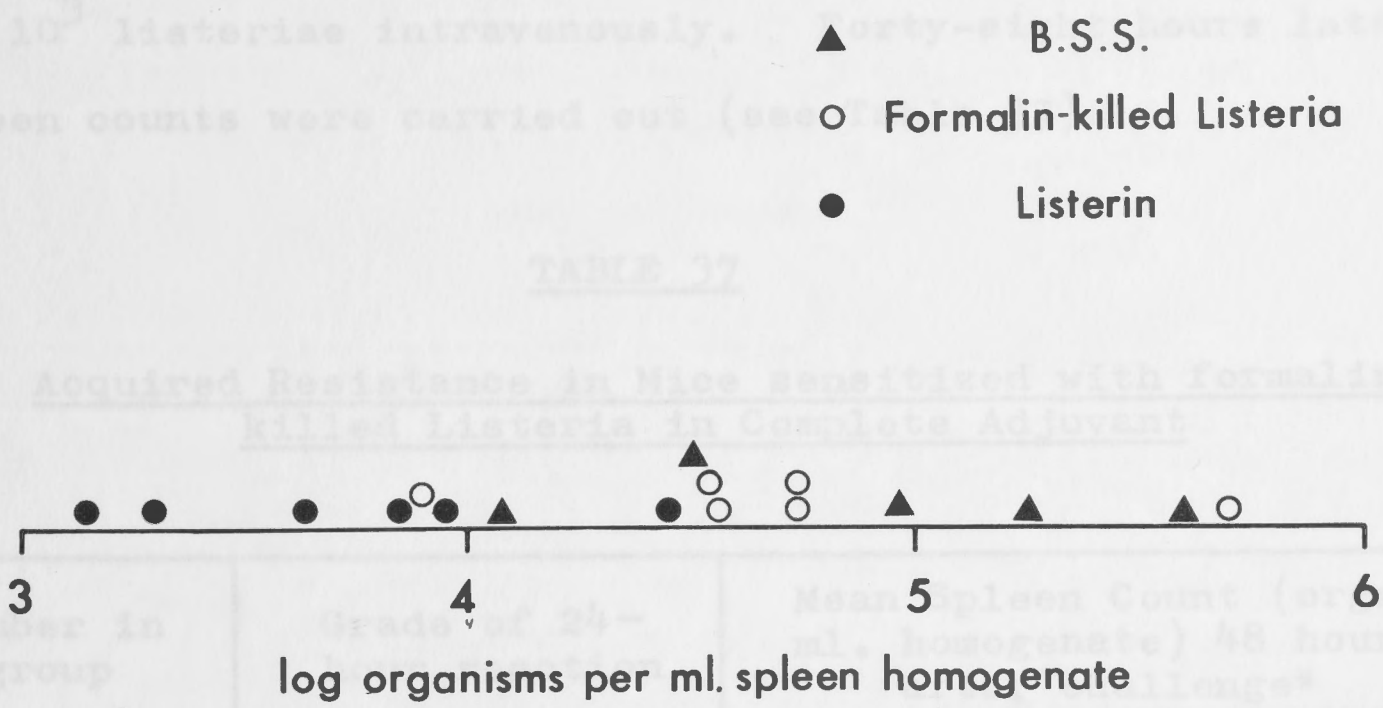


Figure 46.

Individual spleen counts of mice treated with formalin-killed listeriae and challenged with $4 \times 10^3$ viable units of L. monocytogenes.

Hence a certain proportion of these animals must be considered to have been thus sensitized to listerin. The same animals show some degree of acquired resistance to a challenge infection (the difference between the mean spleen count of the "listerin-adjuvant" group and either of the other two is significant at the 1% level). From Figure 46 the "listerin-adjuvant" and "formalin-adjuvant" groups are seen to show virtually no overlap, but the groups are small and it is probably wise to draw no conclusion from this.

In order to examine this correlation more closely a further challenge was carried out. From the group injected with formalin-killed Listeria mice showing distinct delayed

reactions and mice showing none were selected and given $7 \times 10^3$ listeriae intravenously. Forty-eight hours later spleen counts were carried out (see Table 37).

TABLE 37

Acquired Resistance in Mice sensitized with formalin-killed Listeria in Complete Adjuvant

| Number in group | Grade of 24-hour reaction | Mean Spleen Count (organisms/ml. homogenate) 48 hours after challenge* |
|---|---|---|
| 5 | Marked | $2.2 \times 10^5$ |
| 8 | Minimal | $6.8 \times 10^5$ |

* Challenge dose: $7 \times 10^3$ Listeria intravenously.

The difference is again significant at the 1% level; in these counts the intra-group variation is much smaller than usual, differences of this order between means not usually yielding results of this statistical importance. If the distinction between the two groups is valid, the degrees of acquired resistance it indicates is still small when compared with that manifested by the convalescent controls of Table 36, where the ratio of the mean spleen counts of positive and negative controls is 1:8000 (here it is 1:3). The results presented in Tables 36 and 37 are not properly comparable, as the bacterial counts were done respectively 72 and 48 hours after challenge, but ratios of the order of 1:1000 would be expected after 48 hours (see Table 8). Table 36 also shows that the

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TABLE 38

Depression of the Footpad Reaction by Intravenous injection of listerin

| Intravenous injection given | Mean % Increase in Foot-pad Thickness at 24 hours: |
|---|---|
| Nil | 38.4 |
| Listerin, 40 µl. (statim)* | 7.4 |
| " 10 µl. " | 17.7 |
| " 2 µl. " | 21.7 |
| " 40 µl. (+ 6 hours) | 19.5 |
| " 40 µl. (+ 12 hours) | 21.3 |

* Each group received a standard footpad test and then an intravenous injection, either immediately or some hours later. Listerin was diluted appropriately, so that in each case a volume of 0.1 ml. was given.

TABLE 39

Effect of various materials on the footpad reaction

| Material and Route of administration* | Mean % Increase in Foot-pad Thickness at 24 hours: |
|---|---|
| Nil | 33.7 |
| B.S.S. i.v. | 25.0 |
| B.S.S. i.p. | 25.5 |
| PPD (20 µg.) i.p. | 22.9 |
| BCG ($10^{10}$/ml.) i.v. | 19.7 |
| BCG ($10^{9}$/ml.) i.v. | 24.9 |
| Listerin (10 µl.) i.v. | 12.7 |
| " " i.p. | 16.6 |

* The volume injected was 0.1 ml.; the material was diluted where necessary or suspended in B.S.S.

ratio of the mean spleen count of the "listerin-adjuvant" group to that of the "B.S.S.-adjuvant" group is less than 20.

It was decided to abandon this form of attack on the problem, for the reasons given above, and because of other difficulties in the interpretation of the results, to be discussed later.

Desensitization. Desensitization procedures have often been used in investigations of this sort, with no very conclusive results, but as the phenomenon is a feature of tuberculin sensitivity, it was of interest to obtain some information about it in listeriosis. (In the experiments described in this section the mice used were infected with ca. $10^4$ Listeria 3-4 weeks before use).

Preliminary experiments had shown that a temporary desensitization was readily induced by injection of listerin. In the present experiment mice recently infected with L. monocytogenes were divided into groups, which were injected intravenously with the materials indicated in Table 38. The volume given in each case was 0.1 ml, so that the entry "listerin 10 µl." means that 0.1 ml. of a 1/10 dilution of listerin was injected. The results clearly indicate the extreme lability of delayed-type hypersensitivity in such mice, at least in so far as its peripheral expression is concerned. It should be noted also that the controls are not adequate, since the administration of a variety of materials depressed the 24-hour reaction in later experiments. Nevertheless the effect of the intravenous injection of listerin is obviously

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TABLE 40

Statistical Significance of the Results reported in Table 39

| Groups compared | Probability of the difference between these means occurring by chance |
|---|---|
| "Nil" & "B.S.S. i.v." | $< 0.05$ |
| " "B.S.S. i.p." | n.s. |
| "B.S.S. i.v." & "BCG $10^{10}$" | n.s. |
| "B.S.S. i.p." & "PPD i.p." | n.s. |
| "B.S.S. i.v." & "Listerin i.v." | $< 0.001$ |
| "B.S.S. i.p." & "Listerin i.p." | $< 0.05$ |

TABLE 41

Effect of various materials on the Footpad reaction

| Material and route of administration | Mean % Increase in Footpad Thickness at 24 hours: |
|---|---|
| Nil | 28.5 |
| Dialysate medium, i.v. | 25.2 |
| " , i.p. | 20.3 |
| Listerin, 10 µl. i.v. | 14.1 |
| " " i.p. | 11.3 |
| Formalin-killed Listeria, $10^{10}$/ml. i.v. | 4.5 |
| Formalin-killed Listeria, $10^{10}$/ml. i.p. | 5.0 |
| Formalin-killed Listeria, $10^{9}$/ml. i.v. | 3.8 |

The volume of the injection was 0.1 ml. Other materials were diluted or suspended in dialysate medium so that the dose recorded could be given in this volume.

very great; the least depression shown in the Table, that produced by 2 µl. of listerin, is significant well beyond the 0.1% level.

In Tables 39 and 40 further experiments along these lines are reported; in all cases the volume of the desensitizing injection was 0.1 ml.

Thus even the laboratory B.S.S. appeared to have desensitizing properties. In later experiments "pyrogen-free" saline was found to produce no desensitization, but a more suitable diluent was the dialysate medium used for the preparation of listerin. The data reported in Table 41 show that no significant effect is produced by intravenous injection of dialysate medium, whereas by the intraperitoneal route it induces some depression of the footpad reaction. While the difference between the footpad reactions of the groups injected intravenously and intraperitoneally with dialysate medium is slight and not statistically meaningful, it may nevertheless be real, as in general intraperitoneal injections have a greater effect than intravenous, in this and in other experiments.

Over and above the effect of dialysate medium (presumably non-specific, as it does not elicit a footpad reaction, see Table 9), injection of listerin or a suspension of formalin-killed Listeria markedly reduces the 24-hour reaction, even when the quantity injected is equal only to that which will cause a footpad reaction, e.g. 10 µl. listerin or $10^8$ organisms. (See Table 8).

TABLE 42

Duration of Desensitization

| Material injected intravenously* | Interval between i.v. injection and Footpad test | Mean % Increase in Footpad Thickness at 24 hours** |
|---|---|---|
| B.S.S. | 4 days | 34.9 |
| Listerin (40 µl.) | 6 " | 25.1 |
| " " | 4 " | 20.5 |
| " (20 µl.) | 6 " | 22.3 |
| " " | 4 " | 22.6 |

* The volume was 0.1 ml. Listerin was diluted appropriately in B.S.S.

** The least difference shown here between the level of delayed reactivity in the controls (given B.S.S.) and in the listerin-treated mice is significant at the 5% level, the remainder at the 1% level.

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The duration of desensitization was next examined. Mice received quantities of listerin intravenously either 4 or 6 days before all groups underwent footpad test simultaneously. The scheme of injections appears in Table 42, where it is seen that as little as 20 µl. of listerin administered 6 days earlier appreciably reduced the degree of swelling found at 24 hours. How much longer the effect persists is not known. If two footpad reactions are elicited in the right and left hindfeet of the same animal at an interval of 12 days, there is no evidence of desensitization.

In no experiment along these lines was any effect on the bactericidal powers of the spleen perceptible. For example, groups of mice infected 3 weeks previously with $1.6 \times 10^4$ listeriae received 50 µl. and 10 µl. of listerin intravenously; listerin was diluted to 0.1 ml. in dialysate medium and controls received this volume of the diluent. The animals were immediately challenged with $1.2 \times 10^5$ listeriae. The mean spleen counts 48 hours later were, respectively; $3 \times 10^3$, $5.1 \times 10^3$, and $1.8 \times 10^3$ per ml. of spleen homogenate; these differences are not significant.

However, when the degree of acquired resistance was assessed by determination of the $LD_{50}$, the result was most interesting. Mice that had survived a primary infection with $10^6$ listeriae by the intraperitoneal route were given an intraperitoneal injection of the preparation of Listeria antigens (see Chapter I). The dose administered, 0.1 ml., was known to produce complete desensitization for at least

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TABLE 43

Effect of Desensitization on the $LD_{50}$

| Intraperitoneal injection given | $LD_{50}$* |
|---|---|
| Listeria antigens | $1.2 \times 10^{8}$** |
| Dialysate medium | $1.05 \times 10^{7}$ |
| Nil | $2.3 \times 10^{7}$ |

* Estimation of the $LD_{50}$ was performed on groups of 10 mice/dose.

** This value differs significantly from that of either of the controls ($P < 0.003$).

TABLE 44

Effect of Desensitization on Survival of Mice showing * "augmented" delayed reactions

| Group | Survival | Mean % Increase in Foot-pad Thickness at 24 hours ** |
|---|---|---|
| Control | 53% (26/49) | 42.5 |
| "Desensitized" | 94% (65/69) | 47.7 |

* The methods of "augmentation" and of desensitization are described in the text.

** The reaction was measured 24 days before challenge.

four days (cf. the data of Table 42). Controls received 0.1 ml. of dialysate medium or nil. The following day an $LD_{50}$ determination was carried out, as part of the experiment already reported in Table 2. Survival was considerably enhanced in animals that had been desensitized, as indicated in Table 43. The $LD_{50}$ found for the desensitized animals differs significantly from that of either of the controls ($p < 0.003$).

In Chapter IV it was found that the administration of Listeria antigens in incomplete adjuvant some weeks before a primary infection enhanced the development of footpad reactivity considerably and the evidence presented suggested strongly that this 24-hour reactivity was a manifestation of delayed-type hypersensitivity. The effect of desensitization on these animals was investigated. For this purpose they were divided into two groups. One group received 0.05 ml. listerin and 0.1 ml. of the preparation of Listeria antigens, 4 days and 2 days respectively before challenge; it was hoped thus to avoid the possibility of death from systemic shock. While this has not been observed in listeriosis (evidence for the occurrence of delayed shock has not been sought), these mice were thought to be especially at risk. The control group was given no injection (these are the same animals as figure in Table 33). The results of challenge with $3.2 \times 10^7$ viable units of L. monocytogenes by the intravenous route are presented in Table 44.

The two groups do not differ in delayed reactivity

TABLE 45

Relationship between Delayed-type Hypersensitivity and Early Death

| Method of Sensitization | Mean 24-hour Reaction of: | |
|---|---|---|
| | Mice dying within 4 days of challenge | Remainder of group |
| B.S.S. in adjuvant + infection | 29.0 (16)* | 27.1 (21) |
| *Listeria* antigens in adjuvant + infection | 45.4 (11) | 41.9 (38) |

* The figure within brackets indicates the number of animals in the group.

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(readings were made 24 days before challenge). Desensitization with Listeria antigens enhanced survival in a very obvious fashion ($P < 0.0005$). As footpad tests were not carried out in parallel with the challenge, the degree to which the footpad reaction was inhibited is not known, but in view of the earlier findings it seems reasonable to assume that considerable depression would have ensued.

These observations suggested the possibility that systemic shock caused by the injection of large numbers of organisms might be a factor in the mortality from such a challenge. The footpad reactions of mice dying in the first 4 days were therefore compared with those of animals dying later or surviving (Table 45). As only four of the "desensitized" animals failed to survive and these were in no way distinguishable from their fellows with respect to delayed reactivity, this group is not considered here. Neither in animals treated with Listeria antigens before their primary infection nor in the controls given B.S.S. was there a significant difference between the mean 24-hour reactivity of those dying early and of the remainder. There is thus no indication that marked delayed sensitivity is a characteristic of those animals least able to withstand a challenge, nor was any such effect to be detected when footpad reaction was plotted against survival-time (Figures 41 and 43).

The difficulties encountered in the interpretation of desensitization experiments in relation to acquired resistance are highlighted by the observation that animals

undergoing a challenge infection are desensitized. Four weeks after their initial infection with $1.8 \times 10^4$ listeriae mice were footpad tested and immediately afterwards received an intravenous injection of $8 \times 10^4$ listeriae or an equal volume of the suspending medium (0.1% bovine serum albumin). The subsequent footpad reactions appear in Table 46. The difference in 24-hour reactions is highly significant ($P < 0.0001$). This effect is not due to the presence of listerin in the inoculum. The bacterial suspension initially contained ca $2 \times 10^9$ organisms per ml. and was diluted to the point where each mouse received only 0.05 µl. of the original culture medium. Furthermore, when organisms are grown in static culture, as was the practice for the preparation of inocula, the medium has little activity in the footpad test compared with the standard listerin.

Spleen counts were not carried out on these animals. All survived symptomless for ten days. Thus administration of a challenge dose which is to be rapidly and efficiently destroyed nevertheless produces a marked decrease in footpad reactivity. This "anergy" lasts at least 2 days, but has not been followed beyond this point.

TABLE 46

Desensitizing Effect of Challenge Infection

| Material injected intravenously | Mean % Increase in Footpad thickness at: | |
|---|---|---|
| | 3 Hours | 24 Hours |
| Listeria, $8 \times 10^4$ | 15.1 | 11.5 |
| Albumin | 17.7 | 27.4 |

Effect of an Antibiotic on the Development of Hypersensitivity and Acquired Resistance

Miki and Mackaness (personal communication) have shown that mice infected with large doses of Listeria under tetracycline cover develop a high degree of immunity. In these circumstances the organism fails to multiply and the level of the bacterial population of the spleen declines slowly over a period of about 8 days. The effect on the development of delayed sensitivity was not determined. Mice were therefore started on a course of oxytetracycline (2 mg./ml. in the drinking water) 2 days before intravenous infection with $10^7$ listeriae. The antibiotic was administered until day 10 and 12/45 mice died despite it. A control group was given $10^4$ listeriae intravenously in the usual way without antibiotics. On day 15 footpad tests and challenge were carried out, spleen counts being performed 3 days later. The results are reported in Table 47. The differences demonstrated in the levels of delayed-type hypersensitivity and acquired resistance between animals given a large dose of Listeria under cover of oxytetracycline and those infected in standard fashion are both highly significant ($P < 0.001$).

TABLE 47

Development of Delayed-type Hypersensitivity and Acquired Resistance in Mice infected with Listeria under cover of oxytetracycline

| Inoculum and Treatment given | Mean % Increase in footpad thickness at 24 hours | Mean Spleen Count (listeria/ml. homogenate) |
|---|---|---|
| $10^7$ Listeria + oxytetracycline | 11.5 | 45 |
| $10^4$ + nil | 24.8 | $1.8 \times 10^3$ |

The former group has a considerably greater acquired resistance and markedly lower delayed reactivity than the latter.

This can only be considered as a preliminary experiment. The interval of 5 days has been assumed sufficient to allow elimination of the antibiotic from the bodies of the mice, whereas a desirable control would be a group of normal mice treated with tetracycline for the same period. The exact fate of the bacterial population in the spleen should be established by daily counts during both the primary and challenge infections. Despite these reservations the result is considered important.

# DISCUSSION

## I. The Footpad Reaction in Listeriosis

The reaction elicited by *Listeria* antigens in the footpads of mice previously infected with *L. monocytogenes* has been shown to possess most of the features which distinguish delayed-type hypersensitivity.

(a) The reaction is immunologically specific. It is produced only in animals which have undergone infection with *Listeria*; in such animals BCG has no more effect than in normal mice. The desensitization studies provide further evidence of this specificity.

(b) Reactivity is induced only by live suspensions of *Listeria*, not by killed organisms or by listerin (unless an adjuvant containing mycobacteria is used).

(c) The temporal evolution of the reaction is characteristic of delayed-type hypersensitivity. The degree of swelling of the foot is initially slight, but then rises to a peak at 24 to 30 hours after the injection, and is well maintained at 48 hours.

(d) Histological examination shows that listerin induces a striking influx of mononuclear cells, often seen initially in perivascular accumulations, while neutrophils are not prominent at any stage.

In certain features, however, the footpad reaction in listeriosis differs from the tuberculin reaction. As Listeria monocytogenes multiplies in the tissues of the host much more rapidly than does the tubercle bacillus, it isnot surprising that delayed sensitivity should be established earlier (after 4 days, as opposed to 10-14 days). Friedman et al, (1962) could detect skin reactivity in vaccinial infections of guinea-pigs after 5 days, and it is probable that this observation could be duplicated for many acute infections.

It is often stated that in inducing a state of delayed-type hypersensitivity it is important to avoid the intravenous route, subcutaneous or intradermal injection being the method of choice, as the skin plays an important role in the induction process (Lawrence, 1956, 1959; Crowle, 1962). The slight justification for these views in the delayed sensitivities of natural infections has already been discussed, and in listeriosis the experimental evidence points unequivocally to quite the contrary conclusion. Intravenous infection produces a considerably higher level of delayed sensitivity than either subcutaneous or intraperitoneal infection.

In guinea-pigs the degree of hypersensitivity induced by living BCG is independent of the dose given within wide limits (Tolderlund, Bunch-Christensen and Waaler, 1960), whereas in listeriosis of mice this is not the case for either intravenous or intraperitoneal infection. Reduction of the

number of organisms in the inoculum by 100-fold results in a clearly discernible diminution in the mean footpad reactivity. Whether this difference between the two infections is due to factors related to the host or to the parasite is not known.

It may be noted also that these studies confirm the capacity of mice to develop delayed-type hypersensitivity.

II. Delayed-type Hypersensitivity and Acquired Resistance.

The focus of my investigations has been the relationship between these two immunological states. The theme is not novel. The view that delayed sensitivity represents an important mechanism of acquired resistance was first put forward by Römer in 1908 on the basis of his studies on tuberculous re-infection[1]. The negative evidence of repeated failure to demonstrate any correlation between antibodies and resistance to tuberculous infection lent support to this hypothesis, which was further reinforced by the conviction that an immunological phenomenon of such widespread occurrence must have some teleological justification. However proof of the validity of this assumption has not been forthcoming. Römer himself noted that he had demonstrated only "a fairly consistent parallelism" between the states of hypersensitivity and immunity. It is

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[1] This subject was extensively reviewed by Rich in 1951. While presenting a wealth of information, he tends to be uncritical of work supporting his point of view. The evidence offered by his opponents he discusses with an admirable forthrightness.

doubtful if subsequent investigators have been a great deal more successful.

Concepts of the role of delayed-type hypersensitivity in acquired resistance are summarized by Birkhaug (1937) as follows:

1. Delayed-type hypersensitivity and acquired resistance are synonymous.

2. Delayed-type hypersensitivity has an accessory protective function.

3. "....all the known manifestations of allergy, local general and focal, as produced by tuberculin, have the character of harmful systems rather than of protective responses". (Cummings, quoted by Birkhaug).

Innumerable investigations have attempted to uphold one or other of these views.

Clinical and Epidemiological Studies. Many workers have tried to relate the presence or absence of tuberculin sensitivity to the prognosis of the individual known to be suffering from tuberculosis or to the likelihood that a person clinically free of tuberculosis at the time of testing may develop overt infection in the future. Heimbeck (e.g. 1927, 1936) reviewed the incidence of clinical tuberculosis in nurses classified as positive or negative reactors at the start of their training and found that trainees initially negative became tuberculous with greater frequency than those

who were positive. In South African mine workers (Report, 1932) the opposite was found to be the case. In medical students (Diehl et al., 1948) the chronic re-infection type of tuberculosis developed with equal frequency in positive and negative reactors.

Holmes (1915) reported that, in clinical cases of tuberculosis, the level of hypersensitivity was initially high. Reactivity declined if the patient improved and fluctuated or remained unaltered if he did not. In advanced cases sensitivity might rise very high, to decline terminally. On the other hand, Karan and Danford (1934) could show no relationship of any kind between the tuberculin reaction and the patient's clinical course or the type of pathological process manifested by him.

In the negro tuberculosis produces a higher mean level of delayed sensitivity and a more severe disease, i.e. the negro resists tuberculosis less well than the Caucasian. The interpretation of these facts is by no means straightforward. As in the other studies just mentioned, social and environmental factors are neglected in these observations. In a person whose resistance to disease is lowered by e.g. a poor diet, the tubercle bacillus may proliferate unchecked for a longer period, so that he receives in effect a larger inoculum, and is likely therefore to develop a greater degree of hypersensitivity. Nor can one determine whether the degree of exposure to tuberculosis of the groups under investigation is comparable. It is not therefore surprising that the

conclusions arrived at are not unanimous.

The Local Inflammatory Reaction. The only "accessory protective function" that has been directly investigated is the role of the local hypersensitivity reaction. At first sight the Koch phenomenon seems to furnish an obvious example of a protective reaction, as the challenge organisms are sloughed out and the injection site heals. But the Koch phenomenon can only be elicited under certain fairly restricted conditions of primary infection and challenge (Lowenstein, 1913) and in any event it avails the guinea-pig little, since the primary infection will kill it.

In normal animals tubercel bacilli spread from the point of intradermal injection to the regional nodes in 24 hours, whereas in previously immunized animals this process takes about two weeks (Willis, 1925; Krause, 1926). This localization has been ascribed to delayed-type hypersensitivity (the Koch phenomenon does not occur in desensitized animals, Rothschild et al., 1934). While it was shown early that the spread of some types of bacteria, but not all, was retarded if they were deposited in an area of pre-existing inflammation (Pawlowski, 1909; Opie, 1926; Clark, 1929), in the early stages of an inflammatory reaction bacteria tended to be disseminated more widely and rapidly (Rhoads and Goodner, 1931). Krause and Willis had themselves found in 1920 that, if the challenge does was injected into the site of a recent tuberculin reaction, more extensive tuberculosis developed than in controls which had not been skin-tested.

Turk, Allison and Oxman (1962), in an investigation of delayed sensitivity in vaccinia, found that the multiplication of the virus was only slightly reduced in skin which was the site of a delayed reaction, whether this reaction was specific or elicited by an unrelated antigen. (It must be pointed out, however, that the "conventional technique" used to induce delayed sensitivity to vaccinia, combination with an excess of anti-vaccinia serum and incorporation in incomplete adjuvant, is open to criticism, see Section II.E. of the Introduction). Mackaness (1962) challenged mice recently infected with Listeria and could detect no evidence of an inflammatory reaction of delayed, or other, type in their tissues at a time when the animals were rapidly inactivating the organism.

The observations just reviewed make it unlikely that the role of delayed-type hypersensitivity in the processes of acquired resistance is simply to promote localization of the organism or its destruction by an intensified inflammatory reaction.

Experimental Studies. In connection with the evaluation of BCG vaccination, a number of extensive programmes have been carried out in recent years. A group of workers from the U.S. Public Health Service (U.S.P.H.S., 1955 a-e) examined the levels of delayed sensitivity produced by various doses of BCG and H37Ra (a relatively avirulent tubercle bacillus) and compared them with the

immunizing potency as assessed by survival after challenge. In this extensive and carefully controlled study delayed-type hypersensitivity and duration of survival were found to vary in parallel. Groups of guinea-pigs treated with a "strong" vaccine (H37Ra) showed a higher mean skin reactivity and a longer median survival-time than animals injected with a "weak" vaccine (e.g. mixed live and killed BCG). If such groups are further classified into subgroups according to level of delayed sensitivity or duration of survival, then there is a statistically significant tendency for the strong reactors within any one group to survive longer than the weak reactors. The correlation is very slight; in any individual the skin reaction gives little information about the level or acquired resistance.

Jespersen, Weis Bentzon and Magnusson (1962 and 1962a) studied the cutaneous reactivity and survival after challenge of guinea-pigs immunized with a wide range of doses of BCG. Throughout this work, only one group (of 8 animals) demonstrated immunity in the absence of delayed sensitivity; in all the others both responses were either present or absent. Within each group the skin reaction offered no indication of the duration of survival.

Other reports could be cited, but it is clear that in tuberculosis the findings are very similar to those reported here in listeriosis of mice. The position may be stated thus. When groups of animals are infected under various conditions (of dosage and route of inoculation), the resulting mean levels

of delayed-type hypersensitivity and acquired resistance show a positive correlation; almost invariably, the one immune response connotes the other. If, however, these two immunological states are assessed in the individual animal, no such correlation can be found.

In general cutaneous sensitivity seems to wane more rapidly than acquired resistance. Magnusson et al. (1960) found that the immunity of BCG-vaccinated guinea-pigs was unimpaired after nine months, whereas their tuberculin sensitivity has declined considerably. Willis (1928) also observed this. In listeriosis the duration of these immune states has only been followed for four months, by the end of which time footpad reactivity had declined to a greater extent than had immunity.

The state of acquired resistance is the resultant of many factors (see, for example, the discussion of possible mechanisms of immunity in tuberculosis by Raffel, 1960). It is now possible to distinguish a number of phases in this state. In viral infections there is good evidence (reviewed by Wagner, 1963) that interferon plays an important role in the host's defence against a primary viral infection. Friedman et al. (1962) showed that neither antibody nor delayed sensitivity were of major importance in the resistance of guinea-pigs to a primary vaccinial infection. The possibility that the mechanisms effective against an initial and a challenge infection may differ has not been explored in bacterial diseases. In listeriosis the response to the

primary infection is unaffected when the development of delayed sensitivity is depressed by pre-treatment with listerin.

In mice previously infected with L. monocytogenes acquired resistance can be divided into two phases. In the first phase, which lasts ca 2 weeks, the tissues of the mouse are capable of the immediate destruction of a challenge inoculum and the peritoneal cavity contains macrophages which have bactericidal powers in vitro. In the second, anamnestic phase, the challenge organism can multiply for a period before inactivation. During this period the peritoneal cells lack the ability to prevent the intracellular multiplication of L. monocytogenes.

Experiments described above have attempted, (a) to relate individual levels of delayed-type hypersensitivity at various times after infection to the acquired resistance of the animal, assessed within a few days of the footpad test, and (b) to relate the individual level of delayed-type hypersensitivity soon after infection to the animal's response to a challenge infection during the late anamnestic phase (3-4 months after the primary infection). In neither case was any clear-cut correlation evident.

Desensitization. Attempts to dissociate delayed sensitivity and acquired resistance have often utilized desensitization procedures. In many experiments the enormous doses of tuberculin used over long periods killed most of the

animals and the survivors, as Willis noted in 1938, had lost weight, muscle tone and most of their fur and had developed large ulcers. Willis demonstrated more extensive pulmonary lesions in the desensitized animals than in controls after challenge, while Rothschild et al. (1934) found their survival to be unimpaired. Numerous experiments supporting either view-point can be readily quoted.

In any event, the significance of desensitization is difficult to determine. In listeriosis the challenge itself promptly desensitizes the host, while the latter is rapidly eliminating the organism from his tissues. The bactericidal powers of the spleens of convalescent mice are unaltered by desensitization, whereas survival after a challenge is enhanced in a quite clear-cut fashion (this protective effect of desensitization has not been observed in other infections).

This would appear to constitute good evidence that delayed-type hypersensitivity is harmful in certain circumstances. But the experimental data (see Pages 125-6) do not reveal any peak in the death rate at an early stage, as would be expected if systemic delayed shock were a major factor in the death of sensitized animals receiving a large intravenous dose of Listeria. Fenner (1949) found that mice previously infected with ectromelia were more susceptible to intranasal challenge than controls, when the dose of virus was large. Such mice died early, within two days of inoculation, and there was little indication that the virus

had multiplied. Salvin (1955) has reported a very similar phenomenon in Histoplasma capsulatum infection of mice. In both of these conditions delayed sensitivity is known to occur.

The possibility remains, therefore, that enhanced survival after "desensitization" in listeriosis has a different explanation. Mackaness (1964) noted that, in brucellosis of mice, hypersensitivity was more marked early and late in the infection than during the phase of rapid bacterial inactivation. Furthermore, the introduction of additional antigen (by the injection of a further dose of bacteria) was accompanied by a prompt increase in the level of acquired resistance. It is evident that the effect of injection of Listeria antigens on survival is readily explicable on this basis.

In a few experimental situations delayed sensitivity and acquired resistance appear to be at least partially independent of each other. Raffel (1950) could confer no immunity on guinea-pigs by a procedure, injection of tuberculo-protein and wax "D", which induced a state very closely akin to the tuberculin sensitivity of infection. Work reported in this thesis shows that induction of delayed reactivity to listerin by means of complete adjuvant produced a slight but detectable degree of acquired resistance. The method used here to assess resistance is almost certainly much more discriminating. Nevertheless a disparity remains between the levels of the two responses induced.

Repeated skin-testing has been shown (see Section

II.F. of the Introduction) to prevent the decline of tuberculin sensitivity. Magnusson et al. (1960) verified the occurrence of this phenomenon and showed moreover that the level of acquired resistance of the guinea-pigs was not similarly maintained. As skin-testing was last carried out one month before challenge, it is possible that any effect on resistance may have ebbed. In listeriosis "immune" macrophages disappear after ca 2 weeks (v. supra).

The effects of antibiotics on the development of delayed sensitivity in tuberculous animals are not clear-cut; if the infecting dose is large, cutaneous reactivity always appears, but may be weaker than in controls, if the dose is small, it may fail to appear during the period of treatment. The acquired resistance induced by large inocula is unaffected, whereas that induced by small numbers of organisms may be much decreased (Canetti, 1960). In the single experiment carried out along these lines in listeriosis, the level of delayed sensitivity was much lower than that of controls infected with the usual dose of $10^4$ organisms, whereas acquired resistance was considerably increased. If this observation is confirmed, it would appear to indicate that delayed-type hypersensitivity is not an essential factor in resistance.

Augmentation of delayed reactivity by injection of Listeria antigens in incomplete adjuvant before infection (Pages 125-6) had no discernible effect on survival after a challenge infection.

In summary, however, it must be recognised that the elucidation of the interrelations between delayed-type hypersensitivity and acquired resistance has not advanced far beyond the "rather consistent parallelism" noted by Römer. The reasons for this failure can be bluntly stated. We do not know sufficient about either of the immunological state under investigation, and the methods available for their study (this applies especially to delayed sensitivity) are inadequate. Any test for delayed hypersensitivity based on a local reaction, and only such tests can be quantitated readily at present, is a peripheral measurement. We do not know to what extent this reflects systemic reactions. Nor should we forget, while manipulating figures, that "significant" does not necessarily have the same meaning in statistics and biology.

If the mechanism of the local delayed reaction discussed earlier is correct, the cell-type involved in this manifestation of delayed reactivity is the lymphocyte. It is clear that macrophages play an integral part in the cellular immunity of a number of infections. If the postulated cytophilic antibody attaches itself to both types of cells, we are not justified in assuming that we can accurately determine the number of these molecules on one cell by tests involving another. The development of a reliable *in vitro* test for delayed sensitivity is probably the most urgent task in this field.

Assessment of acquired resistance is likewise a complex problem. The least artificial method is probably to

follow the fate of a challenge in the organs of the host, since then the number of organisms can be kept within the sort of limits that might be encountered under natural conditions. In a disease such as listeriosis, where an inoculum of $10^7$ to $10^8$ organisms is required to kill a convalescent mouse, survival studies may easily lead to anomalous results.

Further investigations in this field have a great deal of scope. It is understandable, but regrettable, that immunologists interested in delayed-type hypersensitivity have in the main confined themselves either to diseases such as tuberculosis or brucellosis, where the technical difficulties are extreme, or to the reactivity inducible to simple proteins by means of adjuvants. Very little is known of delayed sensitivity in most acute infections except that it is of frequent occurrence. If delayed sensitivity constitutes an integral part of the acquired resistance to infection, this should be as readily demonstrable in streptococcal disease as in tuberculosis. Certain virus infections, such as herpes simplex, might offer great advantages for these studies, since the number of antigens they contain is far smaller than that present in bacteria, and tissue-culture methods for the assessment of acquired resistance could be developed.

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